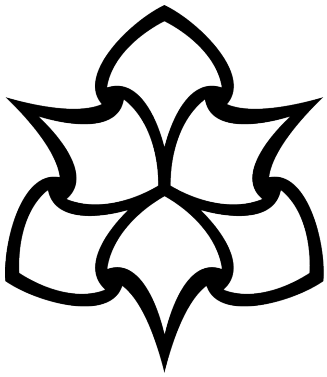

Plasticity of response by saltmarsh plants to changing environmental conditions

R S EDGE

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Plasticity of response by saltmarsh plants to changing environmental conditions

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Abstract:

There is an urgent need to understand how saltmarsh will respond to the changing environmental conditions that result from climate change and other anthropogenic influences. Saltmarsh response to changing environmental conditions is difficult to predict at the ecosystem level as changes depend on the complex responses of species and communities. I investigated the responses of saltmarsh plants at individual, species and simplified community level to altered environmental conditions, including altering flooding regimes to simulate sea level rise using a newly developed Tidal Inundation Machine, and different nutrient conditions to simulate coastal eutrophication. I measured the expression of functional traits in order to relate plant responses to potential ecosystem functioning.

I found that the variation of traits within a species was highly variable, irrespective of treatment and this served to dampen the observed effect of flooding at the community level. The effects of flooding were modified by the addition of nutrients, although this was very context-dependent, and flooding served to modify the intensity and direction of species interactions. I also found that different genotypes had different sensitivities to environmental conditions (flooding and nutrients), even differing in the direction of their response. This has real-world consequences as I found that genetic composition differed between saltmarshes, with variation partially explained by flooding frequency. However, contrary to expectations, restored marshes did not differ from natural sites in their genetic diversity, even two years after restoration.

These experiments were facilitated by the development of the Tidal Inundation Machine that was able to reproduce a true tidal cycle, as well as controlling for nutrient

concentrations, enabling me to study the combined effects of increased tidal inundation and nutrient enrichment.

Overall, I found substantial variation in the responses of individual plants to changes in the environment. Sources of variation included neighbourhood composition, intra-specific trait variability and genotype. Collectively these represent a hierarchy of predictability of responses. This complexity will impact on our ability to predict responses to future change and highlights the need to better understand plants at the individual level before we can predict the response across entire ecosystems.

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0.0 Thesis summary

0.1 Background

Saltmarshes are extremely valuable environments, providing high levels of ecosystem services (Barbier et al., 2011). Large proportions of these habitats have been lost due to historic land reclamation, with some estimates calculating a total loss of up to 50% globally (Marbà, 2009). They are also under increasing pressure from ongoing anthropogenic factors, such as continued reclamation and eutrophication. In light of this, there are global efforts to actively restore these habitats. However, current restoration practices do not produce saltmarsh with equivalent biological characteristics to natural saltmarsh (Mossman et al., 2012). It is extremely difficult to improve restoration practices without a fundamental understanding of the principles that govern saltmarsh ecology. Previous work has identified many of the key drivers of saltmarsh ecosystems such as salinity, elevation flooding and species interactions (Adam, 1990). All of these factors and several others not mentioned do not work in isolation and we still lack a fundamental grasp of the interplay between them. Understanding them requires further multifactor studies and the implementation of novel methods to gleam sufficient understanding to improve current restoration efforts.

0.2 Study design

In this thesis, I first used a traditional glasshouse, multifactorial experiment in order to disentangle the relative effects of tidal inundation and species interactions on the

morphology of saltmarsh plants. Whilst this experiment was successful in gleaming new insight on the interplay between elevation and species interactions, I did identify a large amount of variation which was not accounted for by any of our explanatory variables. Due to the controlled nature of the experiment and inferences made from the literature, I suspect that this variation was likely attributable to changes in the genetic composition of the individuals used in the experiment. Genetics is a particularly understudied area of saltmarsh research. In order to further our understanding, I next improved upon existing technology first developed by Miller and Long, (2015) to create a practical system that better replicated the tidal inundations experienced by a natural marsh as well as controlling for water chemistry. I then used this system to look at how increased nutrient levels would affect the viability and functional trait production of two saltmarsh grasses *Puccinellia maritima* and *Festuca rubra* under a predicted sea level rise scenario, whilst controlling for the genetic identity of the individuals in the experiment. Finally, I looked at one of the study species *Puccinellia maritima* in the field to answer some fundamental questions on how the genetic composition of restored and natural saltmarsh differ and how they may develop over time.

0.3 Outputs

I have been able to disentangle the relative effect of elevation and species interactions between three saltmarsh species with overlapping environmental niches. I was also able to answer some important ecological questions on how the genetic population can influence functional traits and species interactions and how genetic populations of one species differ between restored and natural marsh as well as how such populations may

develop over time. Finally, I was able to design, test and implement a novel piece of equipment for the replication of tides and the control of nutrient levels, successfully using it to answer a crucial question on the role of nutrients in offsetting the negative effects of increased tidal inundation relative to predicted sea level rise. Whilst I only demonstrated one use case, the new tidal inundation machine expands the capabilities and flexibility of previous equipment and has the potential to allow for more controlled and cost-effective studies of tidal inundation and nutrients in wetlands. Importantly, this represents a new and valuable tool for coastal researchers moving forward.

0.4 References

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Chapter 1: Introduction

1.0 Introduction

1.1 Background

Saltmarshes are areas of land inhabited by grasses, herbs and shrubs that are frequently inundated by saline water, usually from a tidal source (Adam, 1990). They are characterised by the salt-tolerant plant species (halophytes) that inhabit them. Saltmarshes are found globally in temperate coastal environments, often being replaced by mangroves in tropical areas. Wetlands, and more specifically saltmarshes, are extremely valuable areas producing a disproportionate level of ecosystem functioning relative to their size; for example, they cover only 3% of the world's surface but provide 40% of annual renewable ecosystem services (Zedler and Kercher, 2005).

Such services include coastal protection, carbon storage, nutrient cycling and habitat provision (Barbier *et al.*, 2011). Coastal protection is provided by saltmarsh vegetation as it helps to dissipate wave energy as it approaches the shore (Pinsky, Guannel and Arkema, 2013). This has a clear economic benefit as studies have shown that for every metre of marsh lost seaward of a sea wall, an exponentially larger sea wall is required in order to provide the same level of coastal protection (King and Lester, 1995). Saltmarsh vegetation also serves to bind sediment, consequently increasing resistance to coastal erosion (Dalby, Allen and Pye, 2006). Saltmarshes act as valuable carbon sinks, trapping carbon within layers of sediment (Macreadie *et al.*, 2017). As well as the physical

processes mentioned, saltmarsh are valuable habitats for many rare and endangered bird species (Spencer, Monamy and Breitfuss, 2009), as well providing important nurseries for commercial fish species (Sheaves *et al.*, 2015). At a very local level, they provide recreational benefits being areas of natural beauty and are regularly used for activities such as dog walking and bird watching (Casagrande, 1997, Adam, 2002).

However, saltmarshes are under threat from a range of factors, including land reclamation, sea level rise and coastal eutrophication, and we risk losing the valuable services they provide as a result. As well as modern threats, human populations have been destroying these areas for thousands of years by building sea walls to block the incoming tide in order to reclaim the land for settlements and agriculture (Adam, 2002). Saltmarsh remains one of the most vulnerable habitats in the world today. Conservative estimates put the global area of saltmarsh at about 5.5 million hectares (Mcowen *et al.*, 2017), but most recent estimates suggest we are losing between 1-2% a year, which as a proportion outstrips the 0.5% figure for tropical rainforest, despite the latter receiving far more media attention (Marbà, 2009). The UK has not been an exception to this global trend. Current best estimates for the extent of saltmarsh cover in the UK is approximately 41,000 ha covering approximately 2000 km of the coastline (Phelan et al., 2011, Mcowen et al., 2017). Historically, one of its largest expanses of saltmarsh was the floodplain of the Humber estuary. It is estimated that the floodplain has been narrowed by over 70%, with the loss of the same amount of saltmarsh (Andrews *et al.*, 2006). The loss of this marsh has been attributed to the increased severity of flooding events in the area, with the most devastating in 2007, causing damage to 8,600 homes and 1,300 business and prompting the implementation of millions of pounds of increased artificial flood defences (Coulthard and Frostick, 2010).

Much of the saltmarsh that does remain, both in the UK and globally, is often hemmed in by sea walls. In the face of rising sea levels, this can cause coastal squeeze as the marsh cannot migrate inland, and the lower elevations of the marsh are lost to the rising sea (Pontee, 2013). However, there remains some debate concerning the true vulnerability of saltmarsh to sea level rise, as rising sea levels have also been linked to an increase in sedimentation, which leads to an increase in relative elevation. This has the potential to offset the relative decrease in elevation due to rising sea levels (Kirwan *et al.*, 2016). In addition to the survival of saltmarsh, there is also evidence that rising sea levels are causing a change in the species compositions and vegetation dynamics of existing saltmarsh (Donnelly and Bertness, 2001; Raposa *et al.*, 2017) and together with loss of these habitats, are predicted to cause a decline in ecosystem service provision (Craft *et al.*, 2009). Section 1.2 "Overview of saltmarsh ecology" discusses the mechanisms by which sea level rise is likely to impact saltmarsh in more detail.

In addition to sea level rise, nutrient enrichment from anthropogenic sources and subsequent eutrophication are also threatening saltmarsh habitat (Boorman, 2003). Nutrient enrichment has been shown to alter saltmarsh vegetation in a variety of ways, from changes in height and stem density as well as losses of below ground biomass (Johnson *et al.*, 2016). Whilst we still lack sufficient evidence to be able to predict the true impact of increased nutrient concentrations in saltmarsh, we do know that they can have potentially disastrous effects. Deegan et al., (2012) showed how increased nutrient concentrations have the potential to damage the structural integrity of saltmarsh by

decreasing below-ground biomass and thus reducing sediment stability, potentially leading to the complete loss of affected areas. Nitrogen is known to be the limiting nutrient affecting mature saltmarsh growth and introduction of nitrogen can change species growth patterns, ultimately leading to changes in species composition (Kiehl, Esselink and Bakker, 1997). In young saltmarsh, such as those that have been newly restored, it is often phosphorous that is the limiting nutrient due to the low levels of organic matter in the soil and additions may have a similar effect to that of nitrogen additions in these areas. In addition, in areas that are already receiving high levels of nitrogen addition, an increasingly likely scenario due to rising coastal eutrophication (Rabalais *et al.*, 2009), phosphorus will also become the limiting nutrient and we need more research to understand its potential impacts in this scenario (Van Wijnen and Bakker, 1999).

In order to combat the loss of saltmarsh habitats, we are now actively restoring land that was historically lost to provide replacement habitat. This restoration is partially driven by legislation, such as the European Habitats Directive, that requires lost habitat to be replaced with an equivalent area that is biologically and functionally equivalent (European Commission, 2000). In the 30 years up to 2014, approximately 1,960 ha of saltmarsh was restored in the UK (ABPmer Online Marine Registry, 2014). Current restoration management is simple and usually involves very little intervention apart from breaching the sea wall and allowing tidal water to flood the previously arable land. The salinity of the water leads to the loss of the freshwater vegetation in the area and it is quickly replaced by salt tolerant species (Garbutt *et al.*, 2006).

Current restoration practices are failing to create saltmarsh with equivalent characteristics to that of natural sites. They lack the morphological characteristics of natural sites having less topographic diversity and, overall, a more homogenous elevation range (Lawrence *et al.*, 2018). This more homogenous environment also leads to sites with homogenous species compositions that lack the diversity and richness of natural sites (Garbutt and Wolters, 2008), with rare species present in natural saltmarsh being absent within restored sites. The end result is that restored sites have significantly different species compositions to that of natural saltmarsh (Mossman, Davy and Grant, 2012).

For many of the ecosystem functions and services, we have insufficient knowledge of the extent of their provision on restored compared to natural saltmarshes. However, as the vegetation communities and structure are different between the marsh types, it is expected that there will also be a difference in marsh functioning. From what comparisons we do have, results indicate that restored sites do not have the same levels of biochemical functioning, with restored sites typically exhibiting levels 25% lower than natural reference sites in the same areas (Moreno-Mateos *et al.*, 2012). Despite these differences there is no consensus on how best to improve current restoration practices. Recently some effort has been made to alter topography but these efforts are usually aimed at altering the hydrology of the site in order to create lagoons for waterfowl and not with the express purpose of creating a hydrological regime comparable to a natural marsh (Tovey, Pontee and Harvey, 2009). There has also been some planting schemes that have had success in creating saltmarsh communities more comparable to natural

sites, but these have been not been implemented in the UK (Zedler, 2000, Callaway et al., 2003). Ultimately, we still lack a detailed enough understanding of the drivers of saltmarsh ecology to design restoration strategies that will create habitat equivalent to natural sites. Preservation of natural habitats is therefore key, as is continued research into saltmarsh ecology.

1.2 Overview of saltmarsh ecology

Saltmarsh forms in areas of high salinity, usually in along a coastline or estuary where low-energy tidal flows allow the build-up of sediment. Sedimentation is a key process that drives the development of saltmarsh. Increases or decreases in sedimentation can cause the development of new saltmarsh or the loss of existing saltmarsh if it is eroded away (Allen, 2000). Broadly speaking, sediment can originate from two different sources, the first being inorganic material brought in externally from rivers or the sea. The second is organic material arising from plants growing on a marsh. We can categorise marshes into organogenic or minerogenic depending on the predominant source of sediment (Allen and Pye, 1992). Whilst factors such as vegetation growth, local hydrology and weather can shift this balance, overarching factors, such micro and macro tidal regimes, means that geographic location plays a large role in determining organogenic or minerogenic status. For example, micro tidal regimes across most of the USA lead to organogenic saltmarsh, whilst western Europe with its comparatively large tidal range is predominately minerogenic (Baptist, de Groot and van Duin, 2016).

The salt tolerant plants that inhabit saltmarshes have adaptations to cope with salinity, many relating to water retention or to actively extrude salt from the tissues to maintain osmotic balance (Flowers and Colmer, 2015). These adaptations allow them to colonise areas that other species cannot. It should be noted that none of the species typically found in saltmarshes in the UK are obligate halophytes and grow well, if not better, in freshwater (Boorman, 1968). The reason they are confined to saltmarsh is that they are typically poor competitors, growing well when transplanted to freshwater areas until they come into competition with freshwater species (Crain *et al.*, 2004).

It is well documented that saltmarsh plant species typically occur in zones along the elevation gradient from low, to mid, to upper marsh (Gray, 1992). Elevation drives this zonation through its determination of the flooding regime, as the frequency and duration of flooding inundation decreases relative to height above sea level. The change in environmental conditions as a result of the different inundation regimes (Armstrong *et al.*, 1985) drives the distribution of species because species vary in their tolerance of the resulting environmental conditions. Salinity is one such variable that acts as an environmental constraint on species distribution, with different tolerances amongst species (Silvestri, Defina and Marani, 2005). Areas of high elevation are often drier and have higher salinity concentrations than regularly flooded lower areas, although this is also heavily modified by hydrological flow throughout a site (Pennings and Bertness, 2000). Another environmental constraint is the oxygenation of the sediment, measured by proxy with redox potential. Areas that are waterlogged tend to have low soil oxygenation, and low redox potential, as oxygen diffuses much slower in water than in air. In addition to the lower oxygen levels in the sediment, low redox potential can lead

to the production of toxic reduced ions (DeLaune and Reddy, 2005). Plant species are vary in their tolerance to waterlogging, low oxygen and toxic reduced ions (Havill, Ingold and Pearson, 1985), leading to zonation (Davy *et al.*, 2011). Small differences in local elevation (microtopography) can alter the flooding regime and the sediment redox potential, and this can affect species distributions (Mossman, Grant and Davy, 2019). This local effect will be more apparent on natural saltmarsh compared to restored sites, as restored sites lack the topographic diversity of natural sites and this may explain some of the observed differences in species composition (Lawrence *et al.*, 2018).

Whilst environmental factors related to flooding predominantly define the possible elevation range of most species, interactions between plant species play a key role in defining the finer structure of marsh communities (Pennings and Callaway, 1992). It is thought that these interactions serve to increase species diversity, with positive interactions allowing species to survive in areas where conditions would otherwise be too harsh (Hacker and Gaines, 2016). They can also shape the development of saltmarsh through facilitated succession. One example of this is the low marsh species *Spartina maritima* trapping sediment to form raised mounds. The change in elevation of these mounds alleviates flooding pressure and the *Spartina* is then displaced by another species *Arthrocnemum perenne*, which is more competitive under the ameliorated conditions (Castellanos, Figueroa and Davy, 1994).

The overall effect of interspecific interactions can be difficult to predict as they can be species-specific, making it difficult to infer a generalised response across entire

saltmarsh without a detailed understanding of the intricacies of interactions between specific species pairs. They can also be difficult to predict as species interactions can change in both intensity and direction depending on a variety of external environmental pressures including salinity, drought/ waterlogging, and grazing type and pressure (Crain, 2008; Nolte *et al.*, 2014; Howison *et al.*, 2015). Overall, we lack sufficient understanding of the interactions between the vast majority of saltmarsh species and how these vary across environmental gradients. As these interactions play such an important role in saltmarsh plant communities, it is vital that we have more studies to understand the details of the interactions between a range of species, and how these interactions may respond to changing environmental conditions.

While we have a relatively good understanding of species composition and its drivers both within and between sites, we know very little about the genetic structure of saltmarsh populations, particularly within the UK. Recent work by Rouger and Jump, (2014) identified distinct populations of *Puccinellia maritima* and *Triglochin maritima* spaced geographically around the UK, and identified two distinct mechanisms for their dispersal. *Puccinellia maritima* populations were separated by barriers to coastal sediment transportation, whilst *Triglochin maritima* populations were isolated by barriers to overland dispersal. For *Puccinellia maritima*, there is also evidence that populations differ within sites, driven by elevation relative to sea level (Rouger and Jump, 2015). It is still unclear what the wider consequences of this genetic structuring will be for saltmarsh species distribution, functioning or response to climate change. Furthermore, we lack similar information for other common saltmarsh species.

Understanding of genetic variation in saltmarsh plants is important because there may be genetic structure related to environmental tolerances, and this may infer future adaptability of populations to changing conditions. For example, variation in response to salinity has been observed between different genetic populations of the saltmarsh species *Borrichia frutescens* (Richards *et al.*, 2010), and similar results have been found in *Plantago coronopus* (Ungar, 1987). *Elymus athericus* populations on the high and low marsh have also been found to be genetically dissimilar to each other (Bockelmann *et al.*, 2003).

Furthermore, plasticity, the phenotypic response of individuals to environmental pressure (Etterson, 2004), may be linked to genetics; although this is a complex field with much debate, it is widely excepted that genetic composition limits plasticity of an individual (Pigliucci, 2005). Plasticity in saltmarsh plants can also help to determine ecosystem service provision by determining the expression of functional traits, and different genotypes have also been shown to express functional traits differently, e.g. *Salicornia europaea* agg. (Ungar, 1987) and *Spartina anglica* (Thompson, McNeily and Gay, 1991). Whilst there is some debate over the definition of functional traits, Violle et al., (2007) define them as "**morpho-physio-phenological traits which impact fitness indirectly via their effects on growth**". By measuring the response of functional traits, researchers have been able to predict the possible response of saltmarsh plants to changing environmental conditions and, importantly, the impact for ecosystem functioning. For example, Minden and Kleyer, (2011) have shown how variations in

salinity, nutrient content and ground water influence the biomass allocations of saltmarsh plants, whilst a variety of different vegetation characteristics have been linked to wave attenuation (Pinsky, Guannel and Arkema, 2013). We are also starting to understand how a response in functional traits to changing environmental conditions may influence saltmarsh in the future. For example, Deegan et al., (2012) showed how a change in biomass allocation resulting in a loss of below ground biomass in response to coastal eutrophication, can lead to a severe decline in structural integrity of a saltmarsh. However, we still need more studies to understand how saltmarsh plants will respond to a variety of environmental changes such as sea level rise and increased nutrient conditions to consider how they will respond in the future.

Understanding the responses of saltmarsh plants, and the responses of interactions between them, to environmental pressures is particularly important in the light of anthropogenic stressors, such as climate change and sea level rise. For example, in addition to causing marsh erosion (Deegan et al., 2012), higher nutrient concentrations as a result of coastal eutrophication have been shown to change the intensity and directions of interspecific interactions, leading to changes in species composition across the marsh (Levine, Brewer and Bertness, 1998). However, disentangling the effects of anthropogenic stressors on saltmarshes is very complex. Rising sea levels will reduce the relative elevation of a marsh if there is insufficient sediment supply (Morris *et al.*, 2009). However, if sedimentation is sufficient a marsh can increase in elevation in line with, or in some cases exceed, rising sea levels (Schuerch *et al.*, 2018). Alterations in the relative elevation of a marsh will alter the salinity and waterlogging pressures, which will also affect the distribution of species and interspecific interactions (Crain, 2008).

Changes to species abundances and composition can also affect marsh elevation gain (Reef et al., 2012) and the amount of organic sediment being deposited on a marsh (Kelleway *et al.*, 2017), which in turn impacts marsh elevation. Increases in atmospheric carbon dioxide may also effect marsh elevation and the response to sea level rise. Experiments have found that elevated CO_2 can increase the rate of surface elevation gain in saltmarshes, possibly indirectly through decreased microbial activity and thus slowing decomposition (Reef *et al.*, 2017). Elevated atmospheric CO_2 also alters the balance of carbon exchange between saltmarsh and the atmosphere, increasing carbon accrual of vegetation and decreasing decomposition rates, whilst simultaneously leading to an increase in CH_4 emissions (Arp *et al.*, 1993). Elevated temperatures associated with climate change can alter the dominance between intertidal species. For example, mangrove invasion into saltmarsh has been linked to higher temperatures (Coldren *et al.*, 2019). Temperature is also one of the key regulators of saltmarsh microbial activity. Increases in temperature of just a few degrees can substantially increase microbial activity, particularly at lower base temperatures (Apple, Del Giorgio and Kemp, 2006). This change in microbial activity can affect oxygenation of the soil as well nutrient profiles, due to changes in levels of Nitrate and Phosphate reduction (King and Nedwell, 1984; Koretsky *et al.*, 2003). In summary, understanding the effects of these multiple anthropogenic stressors on saltmarsh species and species interactions is complex and difficult to disentangle due to the multiple feedbacks between the stressors and the marsh responses. In order to achieve this we need to study the combined effect of stressors and species interactions instead of studying each in isolation.

1.3 Knowledge Gaps

1.3.1 Response to future environmental change

Saltmarshes are under threat from a variety of anthropogenic factors, such as climate change and nutrient pollution (Adam, 2002). The key thread linking these different threats together is that they are likely to cause a change in the environmental conditions experienced by a saltmarsh. We know that changes in environmental conditions can influence saltmarsh ecology in a number of ways, such as a changes in species composition, modification of species interactions, changes in functional traits and changes in genetic composition (Pennings and Callaway, 1992; Levine, Brewer and Bertness, 1998; Donnelly and Bertness, 2001; Rouger and Jump, 2015). However, we do not understand the effects of these changes in sufficient detail, or for enough species, in order to make accurate assessments of how saltmarsh may respond to future changes in climatic conditions. Threats such as rising sea levels and increased nutrient concentrations are unlikely to affect saltmarsh in isolation and so we also need to understand the relative response of saltmarsh plants to these influences in unison.

1.3.2 Specific species interactions

Species interactions are crucial in determining the final species assemblages on a saltmarsh (Callaway, 2006). These interactions can be very complex, being either facilitative or competitive, and change in intensity and direction depending on environmental stress (Pennings and Callaway, 1992). Whilst we recognise the

importance of these interspecific interactions, they have not been described for the vast majority of saltmarsh species. Gaining this knowledge is imperative if we are to understand how saltmarsh will respond at the ecosystem level to changes in environmental pressures. This is particularly important as we know that the effects of climate change, such as sea level rise, is now inevitable and will undoubtedly cause a change in the environmental conditions experienced in coastal areas (Mengel *et al.*, 2018).

1.3.3 Interactions between flooding, nutrients and genetics

Whilst we have a good general understanding of the influences of increased flooding and nutrient levels on saltmarsh plants (Morris *et al.*, 2009; Johnson *et al.*, 2016), we only have very limited information on the combined effects of both (Fox, Valiela and Kinney, 2012; Wong, Van Colen and Airoldi, 2015). As increased flooding is a stressor to saltmarsh plants and increased nutrients are known to alleviate increased stress, it is possible that the combined effects of increased flooding as a result of sea level rise and increased nutrients as a result of increased coastal eutrophication could cancel each other out. However, increased flooding stress can also impair the ability of an individual to take up nutrients (Alam, 1999). This delicate balance between the two competing influences is likely to be different on a species and situational basis and thus we need research targeted at the individual level. The need for this specific, targeted research is even more pressing when we consider the potential for responses to be modified by

genetic compositions of the individuals under study, as several studies have shown that this can also influence saltmarsh plant responses to environmental change through the modification of species interactions (Levine, Brewer and Bertness, 1998; Proffitt *et al.*, 2005). It is difficult to predict what the influence of genetic composition will be in the field as this is a fairly unknown variable in the UK, as we only have information on the genetic populations and their distributions of two species, *Puccinellia maritima* and *Triglochin maritima* (Rouger and Jump, 2015). One of the major barriers to obtaining knowledge on these combined influences is that we cannot easily replicate the intricacies of the natural environment in a controlled setting and instead must rely on large scale field studies such as Deegan, (2002), which whilst impressive are restrictive in terms of scale, time and finances needed to complete. One reason we rely on these studies is that replicating hydrology of a saltmarsh is extremely difficult. The natural tidal cycle and resulting flooding regimes will affect redox potential (Armstrong *et al.*, 1985), and it will also affect the concentrations and dynamics of nutrients, such as the movement of highly mobile nitrogen, through the system (Kuhn, Mendelssohn and Reed, 1999). The complexity of these combined effects is difficult to measure in the field and even harder to replicate faithfully in a laboratory setting.

1.3.4 Response of ecosystem functioning to environmental change

We rely on saltmarsh to provide a variety of important ecosystem services (Boorman, 2003). It is therefore important to understand how future changes in environmental conditions will impact this service provision. A study by Craft et al., (2009) has predicted a reduction in the levels of a number of ecosystems services related to biomass

production, as well as waste management, due to the effects of rising sea levels. Coastal eutrophication is another environmental change with the potential to affect ecosystem service provision of saltmarsh. Deegan et al., (2012) showed how coastal eutrophication could lead to complete loss of saltmarsh with obvious losses in the services they provide. In contrast, less extreme responses such as those found by Johnson et al., (2016) also included increases in plant height and stem density, which have been linked to increased wave attenuation (Möller, 2006). There is clear evidence that the plasticity of saltmarsh plants to adapt to environmental changes could potentially lead to changes in traits that influence ecosystem functioning (Richards, Pennings and Donovan, 2005; Minden *et al.*, 2012; Foust *et al.*, 2016). Studying changes in the response of the traits will help us to predict how saltmarsh plants might respond in the face of changing environmental conditions and what the implications will be for future ecosystem service provision (McGill *et al.*, 2006). However, we still lack research into how the combined effects of different environmental changes, such as sea level rise and nutrient addition, will affect functional trait response, as well as the specific responses of many under-studied species.

1.4 Thesis aims and rationale

In order to better predict how saltmarsh will respond to future environmental change, we need more information on the specific response of the species that have not yet been covered by previous research. We also need more detailed research on the factors that can influence the response of individuals within these species. In order to facilitate

this research, we require the development of machinery that accurately replicates natural environmental pressures, such as tidal flooding and nutrient enrichment. This will allow us to study the effects of increased flooding due to sea level rise and increased nutrient concentrations, in an environment where we can more easily control for external influences such as temperature, rainfall, soil composition and genetic identity of the individuals in the experiment. Finally, in order to understand how any results will translate into real world ecosystems, we need to understand how individuals are distributed within real world environments. Whilst we have good information at the species level, we still have very limited information on the genetic composition of saltmarsh and this will need to be expanded in the future. This thesis has the following aims designed to target these issues:

1. To disentangle the relative effects of flooding and species interactions on saltmarsh plant growth and functional traits.
2. To design a more practical system for accurate replication of tidal inundation and nutrient control in a laboratory setting.
3. To test the combined effect of sea level rise and increased nutrient concentrations on saltmarsh plant functional traits and the differences in response between genotypes.
4. To identify current differences in the genetic populations of restored and natural saltmarsh and to assess changes in genetic development over time since restoration.

1.5 Thesis structure

1.5.1 Overview

This thesis contains five further chapters. Chapter Two will investigate how flooding and intra- and inter-specific interactions influence the growth and functional traits of saltmarsh species. Using a multifactorial glasshouse study, I investigate the interactions of three species that share an overlapping environmental niche. Results from this chapter will increase our knowledge of saltmarsh response to environmental change by giving us a better understanding of how plant communities form in relation to environmental variables. It will also inform us of what the potential influences could be on the ecosystem functioning of the area, as we uncover how their interactions with one another and their environment affect functional traits. Chapter Three details the development and testing of a new piece of equipment that allows better replication of the natural tidal environment in a controlled laboratory setting whilst controlling for nutrient levels in a recirculating water body. This will allow us to test the relative effect of nutrient addition and tidal inundation, which has proven difficult to test for in natural environments. Chapter Four uses this equipment to test the combined effects of nutrient addition and increased tidal inundation relative to sea level rise, on the survival and functional trait production of two saltmarsh grasses, whilst controlling for genetic identity of individuals. Chapter Five will provide vital knowledge on the current state of genetic populations of saltmarsh plants, in the context of restoration efforts. It investigates the difference between populations of *Puccinellia maritima*, a species used in Chapter Three, between natural saltmarsh and restored sites of different ages. These four data chapters (Chapters 2, 3, 4 & 5) all aim to help us better understand how species

respond to changing environmental conditions. The rational for the design of these chapters is summarised in Figure 1.0 below. Chapter Six will provide a synthesis of the main results and discuss their implications for predicting saltmarsh response to environmental change. It will also make recommendations as to future avenues of research based on the results of this thesis.

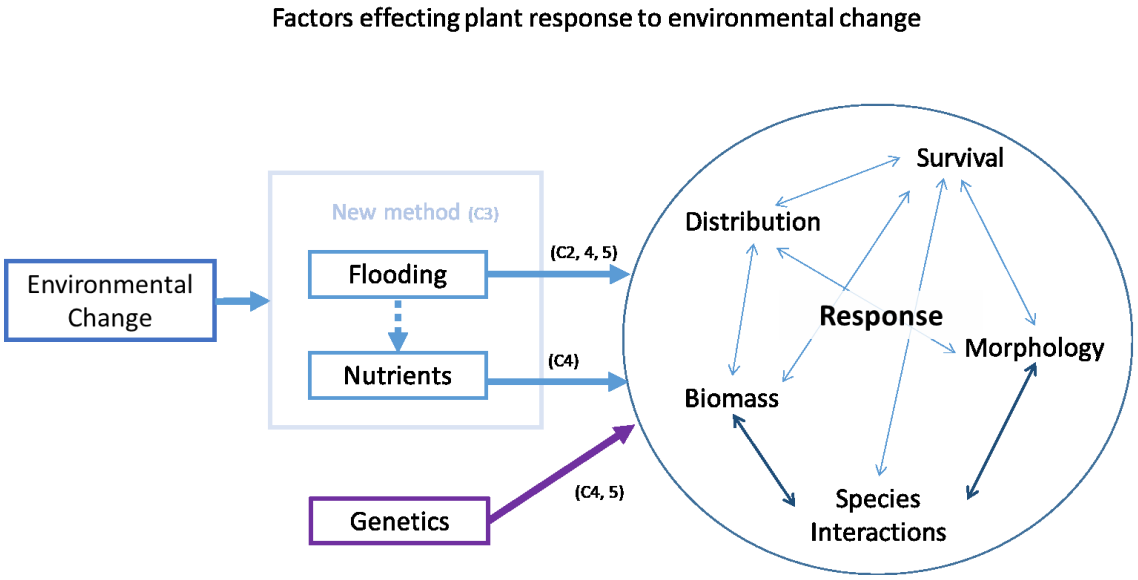

Figure 1.0 depicts a theoretical diagram of the thesis structure and the data contained within. C_n indicates the relevant chapter within the thesis. Moving left to right, all chapters are broadly focussed on plant response to environmental change. Chapters 2, 4 and 5 measured plant response to changes in flooding, with Chapter 4 also including changes in nutrients. Chapter 3 described a new method of simulating changes in flooding and nutrients. Chapters 4 and 5 also included a genetic component to measure how this influences plant response to environmental change. The final circle at the end represents all the response variables contained within the thesis. The dark coloured arrows represent potential interactions between response variables that were directly measured and pale coloured arrows represent interactions that are only inferred from the data collected in the thesis and associated literature.

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Chapter 2: Species interactions modulate the response of saltmarsh plants to flooding

2.0 Abstract

Flooding has long been established as one of the key drivers of species distributions in saltmarsh ecosystems, and also in affecting species morphology. Species interactions are also known to be key drivers of saltmarsh plant distribution and may affect functional traits. However, we do not currently understand the relative influence of these variables. In this study, we used three saltmarsh species whose interactions have not previously been studied but which share an overlapping environmental niche. Using a two-factor glasshouse experiment, we investigated the relative effects of changes in flooding regime and different species interactions on the functional trait response of the individuals and how this translated into overall characteristics of communities. We found that small changes in flooding had a relatively weak effect on functional traits and that species interactions played a much larger role. We also found that flooding served to modify the response to species interactions. We found that the combined response was species and situational specific. We also found a lot of variation in functional traits not attributable to our study variables that was most likely due to differences in genetic composition. This adds to a growing body of research into the relative impact of flooding and species interactions in saltmarsh and also highlights a potentially fruitful avenue for research into the effect of genetic composition within saltmarsh plants.

2.1 Introduction

Functional traits provide a powerful tool to understand how a plant interacts with its environment. There are several terms often used to describe functional traits in the literature but they can be broadly categorised into "response traits" and "effect traits", with response traits being how a plant responds to a change in the environment and effect traits being how a change in the plant affects the environment. As well as being affected by the environment, response traits can also be influenced by inter- and intra-specific interactions (Venterink and Güsewell, 2010). These interactions are thought to play a key role in the shaping of community assemblages and thus also have a large impact on the ecosystem functioning (Kraft, Godoy and Levine, 2015). As cumulative human impacts lead to ever increasing environmental change, most notably in the effects of global warming, functional traits provide a way to study both the physiological responses of species and how changes in these may influence wider ecosystem processes (Diaz and Cabido, 2006).

Saltmarshes have historically been in decline due to anthropogenic impacts, mainly land reclamation. The loss of these areas is important as saltmarshes provide high levels of ecosystem services, such as protection from coastal erosion and carbon sequestration (Barbier *et al.*, 2011). We now recognise the value of these habitats and they are being actively restored, with approximately 2000 hectares of saltmarsh being created in the UK in the last 25 years (ABPmer Online Marine Registry, 2014). Despite these efforts, our understanding of saltmarsh functioning, the role that individual species play in that functioning, and how this is mediated by environmental conditions is poor.

Furthermore, we do not understand how global environmental change will affect community composition and the functional traits, and hence the services these habitats provide. Functional trait-based approaches provide a way to interpret species response to change as well as the associated impacts to ecosystem functioning (Bouma *et al.*, 2005; Minden *et al.*, 2012; Bardgett, Mommer and De Vries, 2014).

The distribution and diversity of saltmarsh plant species is largely dictated by elevation gradients that cause variation in the frequency and duration of tidal flooding (Davy *et al.*, 2011). Even small changes in elevation can have substantial impacts on other environmental conditions, such as waterlogging, and therefore subsequent species assemblages (Bertness and Ellison, 1987). Flooding is directly linked to soil oxidation and this has been shown to affect the species present, both in their survival and functioning (Pezeshki and DeLaune, 2012). Differences in species assemblages have been linked to changes in ecosystem function, with a study by Ford et al., (2016) demonstrating that increased biodiversity leads to greater soil stability in saltmarsh. Diversity within species is also likely to impact ecosystem function. Both species assemblages and the morphology of the species present within a community can be shaped by environmental conditions. For example, the saltmarsh species *Suaeda maritima* can show large amounts of phenotypic plasticity in relation to levels of redox potential, particularly during its early growth (Wetson *et al.*, 2012).

Although the link between biodiversity, and the factors that affect it such as flooding regime, and ecosystem functioning has been demonstrated in some systems, we

currently have limited evidence in saltmarshes. Furthermore, we do not understand the role that individual species, and interactions between them, play in mediating biodiversity-ecosystem functioning. Research has previously uncovered both competitive and facilitative interactions between saltmarsh plants (Luo et al., 2010, Castellanos et al., 2006, Callaway et al., 2000), which have the ability to shape species' distributions (Pennings and Callaway, 1992). In order to understand the mechanisms behind how these interactions shape species assemblages, we need to understand how they influence plant growth, and thus affect ecosystem functioning. Studies such as Wardle and Peltzer (2003) have shown how simple pairwise interactions can affect traits important to ecosystem functioning such as biomass allocation. There is also evidence that even weak pairwise interactions can translate into large differences at community level (Berlow, 1999). However, it is often difficult to predict the true effect of species interactions on functioning of entire ecosystems as multiple species growing together can illicit different effects. Our current theoretical framework, as summarised by Cadotte (2017) states that species assemblages contribute to ecosystem functioning via niche complementarity and somewhat opposing competitive differences. The same study tested this framework and showed that by comparing functional traits of species they could predict the overall level of functioning of different assemblages. They found complementarity was highest in assemblages that had the greatest dissimilarity across a wide range for traits and selection effects where stronger when there was low dissimilarity between traits.

We currently lack detailed information on how species interactions affects functioning of saltmarsh. Work by Ford *et al.*, (2016) found that increased species diversity could be

linked to greater root mass and soil stabilisation, although they did not identify the specific interactions that lead to this. This omission is particularly important as saltmarshes are characterised by low richness of salt-tolerant species. This diversity is further diminished when we consider that areas of saltmarsh are striated along elevation gradients, leading to areas of marsh containing a smaller subset of these species; for example, data from Mossman et al., (2012) found that median species richness in a 0.5m by 0.5m quadrat was three. This low species richness increases the relative importance of interactions between just a few species pairs, and likely increases the importance of individual species to ecosystem functioning. This is further complicated as we have evidence that the strength of this interactions can potentially change across this environmental gradient. For example, the strength of the competitive interactions between two saltmarsh species, *Salicornia virginica and Triglochin concinna*, was found to change along stress gradients, leading to a change in biomass partitioning (ratio of above and below ground biomass) for each species (Morzaria-Luna and Zedler, 2014). Identifying the effect of specific interactions will therefore be crucial in the future as we look to make predictions on ecosystem functioning in response to environmental change.

This study is a two-factor glasshouse experiment to investigate the response of functional traits in three common saltmarsh species to flooding, a key environmental driver of saltmarsh plant distribution that is expected to intensify with sea level rise (Mengel et al., 2018), as well their response to inter- and intra-specific interactions. Our three study species, *Aster tripolium*, *Plantago maritima* and *Triglochin maritima*, were selected as they occur at mid elevations of the marsh and overlap in part of their niche

(Sullivan *et al.*, 2018). *Aster tripolium* grows across the largest range of environmental conditions on the marsh, whilst *Plantago maritima* and *Triglochin maritima* grow across a comparatively narrower range but share almost identical environmental niches. The overlapping niches of the species makes them good candidates to investigate the effects of inter-specific interactions.

This aim of this study is to disentangle the relative effects of flooding and species interactions on saltmarsh plant growth and functional traits. We have identified three research questions that will allow us to achieve this aim and these form the basis of this study.

1. Does flooding effect functional traits?

2. Does species composition effect functional plant traits?

3. Are there interactive effects of species composition and flooding on functional traits?

2.2 Methodology

2.2.1 Experimental design

We investigated the responses of three saltmarsh plant species, *Triglochin maritima*, *Plantago maritima* and *Aster tripolium*, to flooding and species composition treatments in a fully factorial glasshouse experiment. Pots (diameter 25 cm, volume 5L) were filled with a mixture of sand and loam (ratio of 1:1), and planted with six nursery-grown

individuals (grown by British Wildflowers, North Burlingham, Norfolk). Seeds were provided by multiple members of Manchester Metropolitan coastal research group and collected from a variety of sites across the UK, and seeds from different populations were mixed. Individuals were potted in one of the seven possible planting combinations of the three species. These were the three single species combinations, three two-species combinations and one three-species composition (Figure 2.1). Each species combination was replicated 16 times; eight of these replicates were assigned to the flooded treatment and the other eight to the unflooded control (totalling 112 pots and 672 Individual plants; Figure 2.1).

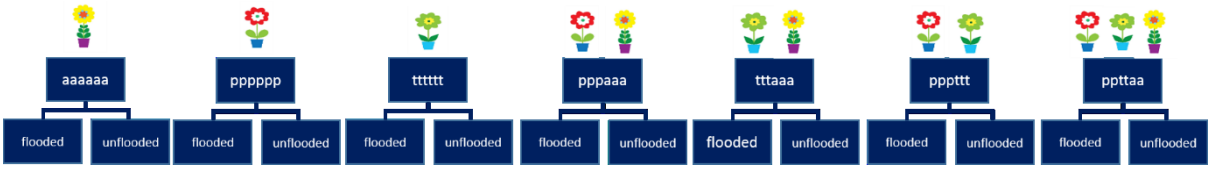

Figure 2.1 Design of two factor experiment. First row of plant pictures is a visual aid, second row of text illustrates the different species combination treatment and number of replicates of each species. a = *Aster tripolium*, p = *Plantago maritima*, t = *Triglochin maritima*

Pots in the flooded treatment were placed in 10 cm deep trays that were filled to a depth of 7 cm with saline water (concentration 50% seawater) for the duration of the experiment. Plants in the unflooded treatment were watered to saturation every three days and allowed to drain freely. Pots were randomly distributed throughout the greenhouse and were grown for five months between December 2017 and May 2018. A 12-hour dark-light cycle was provided by four grow lamps distributed evenly throughout the greenhouse. Temperature was kept above 15 ˚C throughout the experiment. Humidity was not directly controlled but the greenhouse was well ventilated.

After the growth period, we measured seven plant traits and three whole pot measures. The seven plants traits measured were height (mm), width (mm), presence of reproductive material (flowers or seed heads), number of leaves, specific leaf area, above ground biomass (mg), and root mass (mg). The whole pot measures were side on surface area (mm^2) and top down canopy cover (mm^2). Three other whole pot measures were derived from the measurements on the individuals within the pot. These were total biomass (mg), above ground biomass (mg) and root mass (mg).

2.2.2 Individual plant measurements

Plant height, width, number of leaves, specific leaf area, above ground biomass and root mass were measured as per the methods described in Cornelissen et al., (2003), and are briefly summarised below. Plant height was measured from the base of the plant to the maximum height of the plant, excluding any reproductive structures. Width was measured at the widest part of the plant. The number of live leaves were counted and from these, three average leaves were selected to be processed for specific leaf area. Each of these leaves were placed flat on a flatbed scanner and scanned at 300 dpi with a resolution of 4961 x 3508, and from the resulting images leaf area was calculated using a custom protocol in ImageJ (Appendix 2.1). The scanned leaves were then placed in individual paper bags for drying. The rest of the above ground material of each plant was removed and placed in paper bags for drying. Soil was washed from roots through three graduating sieves, the finest being 20 μm, then roots were collected with tweezers

and placed into a paper bag. The aboveground and belowground material, and three individual scanned leaves were dried in an oven at 90 ˚C for 24 hours and then weighed. Specific leaf area was calculated by dividing the one sided area of a leaf by its dry mass.

2.2.3 Pot level traits

Pot-level biomass traits, total biomass, root mass and above ground biomass were calculated for each pot by adding together the measurements of every individual in the pot. Two traits, top-down surface area and side-on surface area, were also calculated for each pot, which provide an indication of plant cover and density. Top-down surface area measured the amount of plant material when viewed from directly above to give a measurement of canopy cover. Side-on surface area measured the total area of plants presented at a 90-degree side-on angle to give a measure of the plant area that could be presented to a wave front. The methodology for this has been adapted from field studies by Möller, (2006) who used side on photographs to determine vegetation density, its relationship with other traits measures and their effect on wave attenuation.

Top down surface area and side on surface area are novel methods consisting of image processing of standardised digital photography and a detailed description of the methodology can be found in Appendix 2.1. Images were obtained for side-on surface area by placing the whole pot on a custom-built white stand against a white background mounted 5 cm from the back of the pot. A picture was taken on a tripod-mounted SLR camera at a 90-degree angle perpendicular to the pot from a distance of 2 m. The camera was focused on the centre of the pot and kept the same for all pictures.

Dispersed room lighting was used to avoid over-exposure. To measure top-down canopy cover, the pot was then placed onto a dark background and an image taken from 1 m directly above the pot, using the same image settings as previously described. Custom protocols were developed to calculate leaf area, side on surface area and top down canopy cover from these images, using the free open source image-processing software ImageJ (Schindelin *et al.*, 2009). The protocols have been packaged into an easy to use macro for ImageJ that can be run directly in the ImageJ interface. Further details can be found in Appendix 2.1.

2.2.4 Data analysis

All analysis was conducted in R Studio (R Studio Team, 2019). A small number of individuals died during the course of the experiment and these individuals were excluded from the species level analyses. Pots where one or more individuals died were included in pot-level analyses, with the weight of the dead plants counted as zero, as the zero measure was a true reflection of the biomass in the pot. A further three percent of the total samples were damaged during storage and before processing. The resulting missing measurements result in a range of sample sizes for the individual measurements (Table 2.1a). Pots missing individual biomass measurements were excluded from all pot-level statistical analysis. This resulted in differences between sample sizes for each treatment. Table 2.1a and 2.1b contain the full list of sample sizes for the species and pot level treatments respectively.

Table 2.1a List of sample sizes available for statistical analysis for each functional trait for each species. The maximum possible sample size is 224

	Height	**Width**	**Number of leaves**	**Above ground biomass**	**Below ground biomass**	**Total Biomass**	**Specific leaf area**
Aster tripolium	207	207	218	199	203	196	188
Plantago maritima	222	222	223	216	216	213	197
Triglochin maritima	224	224	219	207	213	201	196

Table 2.1b List of sample sizes available for statistical analysis for each functional trait in each species composition and flooding treatment. The maximum possible sample size is eight.

	Side-on area	**Top down area**	**Above ground biomass**	**Below ground biomass**	**Total biomass**
A Flooded	8	8	8	8	8
A Unflooded	8	7	6	8	6
P Flooded	8	8	6	6	4
P Unflooded	7	6	8	8	8
T Flooded	8	7	7	6	6
T Unflooded	8	6	4	5	4
PA Flooded	7	7	5	7	5
PA Unflooded	6	8	6	8	6
PT Flooded	8	8	8	7	7
PT Unflooded	8	7	7	7	7
TA Flooded	6	8	5	6	4
TA Unflooded	8	7	5	6	3
PTA Flooded	8	7	6	7	5
PTA Unflooded	7	7	5	7	5

Differences in the survival of species between flooding treatments were investigated with binomial tests for each species. A chi-squared test was performed for each species to assess the difference in number of individuals with reproductive structures between the flooded and unflooded treatments, and separately for the species composition treatment. Few individuals in the experiment produced any form of reproductive structure, so there was insufficient data to perform any analysis of the interactions between flooding treatments and species composition treatments on the number of reproductive structures.

The remaining traits were analysed using two-way ANOVAs, where flooding treatments and species composition treatments were two fixed factors and there was an interaction term between them. A significant interaction term in our model denotes a difference in

response of a trait to the species composition treatment depending on whether it was also the flooded or unflooded treatment. Post-hoc pairwise comparisons were performed to assess statistically significant differences between species composition and flooding treatment combinations. This methodology allowed us to investigate whether plant traits were affected by species composition and flooding as individual factors as well as whether species composition could affect responses to the flooding treatment.

In all our analysis, flooding treatment consisted of two categories, flooded and unflooded. Species composition consisted of the groups described in the experimental design outlined above. Throughout the results we use the following shorthand to describe the different species compositions; **A** *Aster* grown as a monoculture, **P** *Plantago* grown as a monoculture, **T** *Triglochin* grown as a monoculture, **PT** *Plantago* and *Triglochin* grown together, **PA** *Plantago* and *Aster* grown together, **TA** *Triglochin* and *Aster* grown together, and **PTA** all three species grown together.

2.3 Results

2.3.1 Survival

Nineteen individuals planted at the beginning of the experiment died by the time sampling occurred; 17 *Aster tripolium* individuals and two of *Plantago maritima* died, and there were no deaths occurred of *Triglochin maritima*. For *Aster tripolium*, there was no significant difference in survival between flooding treatments with eleven deaths

in the flooded treatment and six deaths in the unflooded treatments (binomial test p = 0.33). There was insufficient sample size to test for differences in survival of other species.

2.3.2 Reproductive structures

Only 32 individuals across all species produced reproductive structures. Of these 32 individuals, two were *Aster tripolium*, 25 were *Plantago maritima* and five were *Triglochin maritima*. There was no significant difference in the number of reproductive structures between the two flooding treatments for any of the species: *Aster tripolium*, no individuals had reproductive structures in the flooded treatment and two in the unflooded (χ^2 = 0.505, df = 1, p= 0.478); *Plantago maritima*, eight in the flooded treatment and 17 in the unflooded (χ^2 = 2.882 , df=1, p=0.090); *Triglochin maritima*, three in the flooded treatments and two in the unflooded treatment (χ^2 = 0, df = 1, p= 1). There was a significant difference in the number individuals of *Plantago maritima* containing reproductive structures between the different species compositions, with PA having more structures than expected; seven individuals had structures in P monocultures, twelve in PA, two in PT and four in PTA compositions (χ^2 = 13.147, df = 3, p = 0.004). There was no difference in number of individuals containing reproductive structures between the different species compositions for either *Aster tripolium* (χ^2 = 1.514, df = 3, p = 0.679) or *Triglochin maritima* (χ^2 =5.387, df = 3, p = 0.146).

2.3.3 Biomass Traits

2.3.4 Total biomass

Aster tripolium had significantly more total biomass in the unflooded treatment compared to the flooded treatment ($F_{1,186} = 5.705$, $p = 0.018$), but there was no response of total biomass to flooding in the other two species (*Plantago maritima* $F_{1,205} = 0.518$, $p = 0.473$; *Triglochin maritima* ($F_{1,193} = 2.362$, $p = 0.126$). In these two species, the predominant factor affecting total biomass was species composition. Both *Plantago* ($F_{3,205} = 17.651$, $p<0.001$) and *Triglochin* ($F_{3,193} = 7.955$, $p<0.001$) showed significant differences between species compositions, whilst there was no difference in total biomass between the different species compositions of *Aster* ($F_{3,186} = 2.310$, $p = 0.078$). Furthermore, pairwise comparisons revealed that *Plantago* and *Triglochin* had more total biomass when grown together compared to the other treatments and less biomass when grown with *Aster* (Figure 2.2; all pairwise comparisons are given in Appendix 2.2). *Plantago maritima* individuals had higher total biomass in the PT unflooded treatment than all the other treatments except PT flooded and PTA flooded (Figure 2.2). *Plantago maritima* individuals in the PT flooded had also had significantly more total biomass than in the P flooded, PA flooded and PA unflooded. *Triglochin maritima* individuals in the PT unflooded had significantly more total biomass than in the TA flooded and TA unflooded, whilst *Triglochin* individuals in PT flooded had significantly more total biomass than in TA flooded.

2.3.5 Above ground biomass

Overall, we found a similar response of above ground biomass as total biomass between the flooding treatments and species composition treatments. *Aster tripolium* was again the only species to show a response to flooding treatment, having significantly more total biomass in the unflooded treatment ($F_{1,189} = 5.872$, $p = 0.016$). As in total biomass, there was no significant effect of flooding on above ground biomass for either *Plantago maritima* ($F_{1,2\text{-}8} = 1.034$, $p = 0.311$) or *Triglochin maritima* ($F_{1,199} = 3.647$, $p = 0.058$). *Plantago* ($F_{3,208} = 8.974$, $p < 0.001$) and *Triglochin* ($F_{3,199} = 6.272$, $p<0.001$) both showed a significant response to species composition treatments. Composition treatments containing *Plantago* and *Triglochin* had the most above ground biomass, whilst pots containing *Triglochin* or *Plantago* with *Aster* had the least (Figure 2.2). For *Plantago maritima*, individuals in the PT flooded and PT unflooded had significantly more above ground biomass compared to those in the P flooded and PA flooded, whilst individuals in PTA flooded also had significantly more above ground biomass than in the PA flooded. For *Triglochin,* individuals in PT flooded had significantly more above ground biomass than in T flooded, TA flooded, TA unflooded and PTA unflooded. In contrast to total biomass, where there was no response to composition treatments by *Aster*, there was a significant difference in above ground biomass of *Aster* individuals between the different species composition treatments ($F_{3,189} = 3.943$, $p = 0.009$). This was driven by an interaction between flooding and one of the species composition treatments (Figure 2.2, solid line; $F_{3,189} = 2.586$, $p = 0.054$). *Aster* individuals in the PA flooded showed a significantly different response to PA unflooded, with those in the PA flooded having the smallest above ground biomass of all the treatments and PA unflooded had the largest.

2.3.6 Root mass

There were some differences in responses of root mass compared to that of total or above ground biomass. In *Aster* there was no response in root mass to either the flooding treatment ($F_{1,192}$ = 2.77, p = 0.092) or the species composition treatment ($F_{3,192}$ = 0.845, p = 0.471). *Plantago* responses remained similar to those seen in above ground and total biomass, although the effects of species composition appeared to be more muted (Figure 2.2). There was no response of root mass to flooding in *Plantago* ($F_{1,208}$= 0.077, p = 0.782), but a significant response to species compositions ($F_{1,208}$ = 5.303, p = 0.002). Pairwise comparisons revealed that the only significant difference was individuals in the PT flooded having more root mass than those in the PA flooded. *Triglochin* showed a significant response to the flooding treatment having more root mass in the unflooded treatment ($F_{1,205}$ =16.837, p <0.001), as well as a significant response to species composition ($F_{3,205}$ =3.151, p = 0.026). Pairwise comparisons showed that, similar to the other measures of biomass, *Triglochin* individuals in pots containing both *Plantago* and *Triglochin* in the absence of *Aster* were larger; individuals in the PT unflooded and PTA unflooded were larger that T flooded, TA flooded and PTA flooded. There was also a marginally significant interaction between flooding and species treatments for *Triglochin* ($F_{3,205}$= 2.651, p = 0.050, solid line in Figure 2.2). Individuals in the PTA unflooded had the most root mass of all the treatments whilst those in the PTA flooded had the least.

2.3.7 Root to shoot ratio

There was no significant effect of flooding on root to shoot ratio of *Aster* ($F_{1,193}$ = 0.0001, p=0.90), *Plantago* ($F_{1,212}$ = 0.049, p =0.626) or *Triglochin* ($F_{1,220}$ = 3.601, p = 0.068). There was also no effect of composition for both *Aster* ($F_{3,193}$ = 0.383, p = 0.765) and *Plantago* ($F_{3,212}$ = 0.585, p = 0.823). For *Triglochin*, there was a significant effect of species composition ($F_{3,220}$ = 2.788, p = 0.042), and a significant interaction between species composition and flooding ($F_{3,220}$ = 2.911, p = 0.035), with PT unflooded being significantly larger that T unflooded.

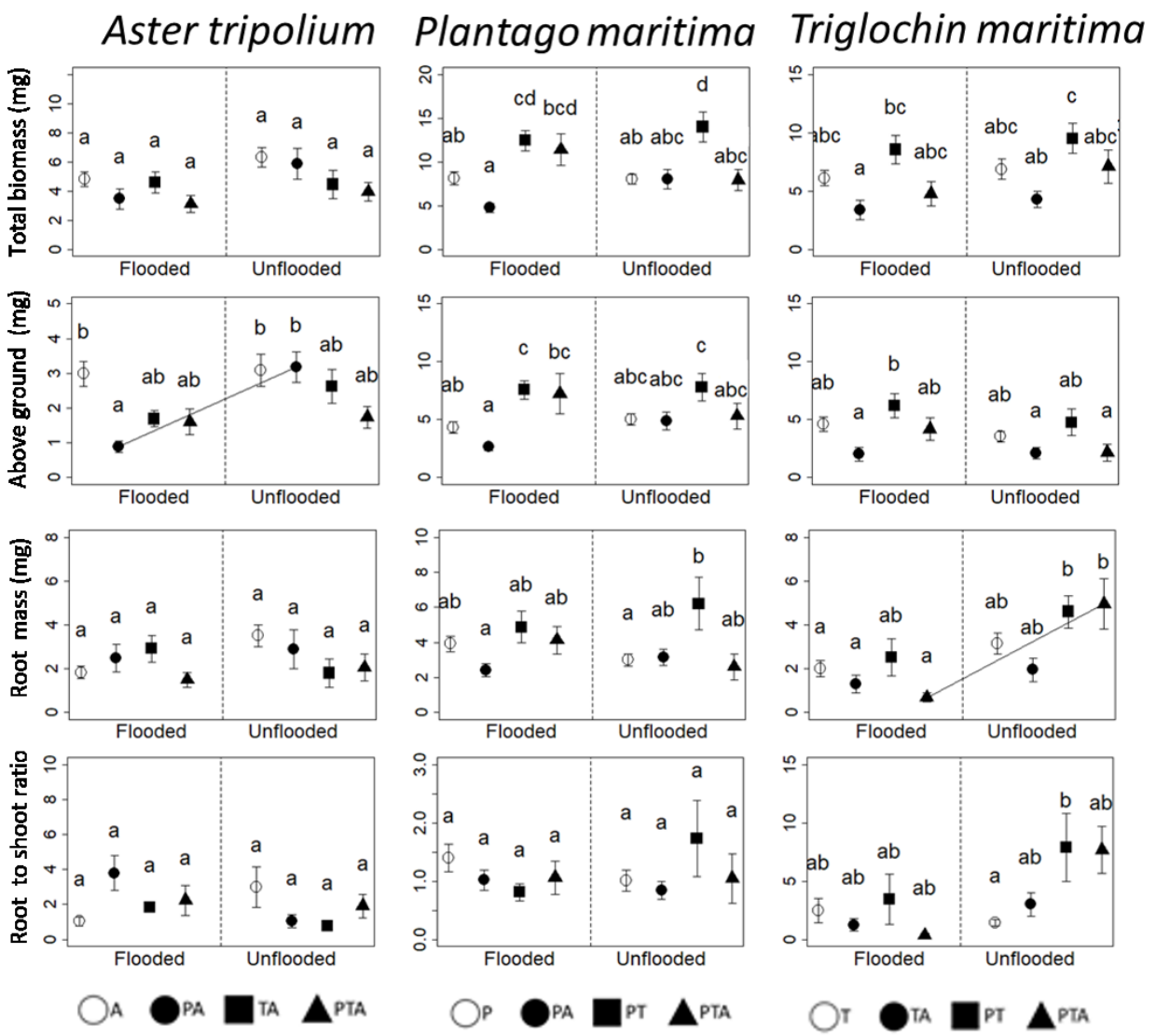

Figure 2.2 Biomass traits at species level. Columns of panel left to right Aster tripolium, Plantago maritima, and Triglochin maritima. Rows top to bottom, total biomass (mg), above ground biomass (mg), and root mass (mg). Letters in the plot refer to statistical differences derived from the Tukey post hoc test following a two-way ANOVA with interaction term. Lines between points denote significant interactions between species composition and flooding treatments. Legend key A = Aster tripolium only, P= Plantago maritima only, T= Triglochin maritima only, PA = Plantago maritima and Aster tripolium grown together, TA= Triglochin maritima and Aster tripolium grown together, PT Plantago maritima and Triglochin maritima grown together. PTA = Plantago maritima Triglochin maritima and Aster tripolium grown together.

2.3.8 Physiology traits

2.3.9 Height

Aster tripolium showed no response in height to either the flooding ($F_{1,194}$ = 0.745, p = 0.389) or species composition treatments ($F_{1,194}$ = 2.369, p = 0.072). *Plantago maritima* and *Triglochin maritima* were both taller in the flooded treatments compared to the unflooded treatments (Plantago $F_{1,214}$ = 43.692, p <0.001; Triglochin $F_{1,211}$ = 5.071, p = 0.026). *Plantago* and *Triglochin* also both showed a response in height to the species composition treatments (Plantago $F_{3,214}$ = 4.228, p = 0.006; Triglochin $F_{3,211}$ = 5.636 p<0.001). Pairwise comparisons and the interaction term within the ANOVA analysis (Plantago $F_{3,192}$ = 9.083, p <0.001; Triglochin $F_{3,211}$ = 9.083, p = 0.001) showed that these differences were driven by a single response of one species composition to the flooding treatments (Figure 2.3). For *Plantago,* this was that individuals in the P flooded were taller than those in the P unflooded, and *Triglochin* individuals in the PTA flooded were significantly taller than PTA unflooded.

2.3.10 Width

There was no significant response in the width of *Aster* or *Triglochin* to the flooding treatment (Aster $F_{1,194}$ = 0.05, p = 0.81; Triglochin $F_{2,211}$ = 1.38, p = 0.24). *Plantago* was significantly wider in the flooded treatment compared to unflooded treatment ($F_{1,214}$ = 13.891, p<0.001). Both *Plantago* and *Aster* showed no response in width to the different species composition treatments (Plantago $F_{3,214}$ = 2.162, p = 0.094; Aster $F_{3,194}$ = 2.393,

p = 0.070). *Triglochin* did show a significant difference in width between the different species composition ($F_{3,211}$ = 8.244, p<0.001), as well as significant interaction between flooding and species composition treatments ($F_{3,211}$ = 7.501, p <0.001, Figure 2.3). This was the same interaction as found in height, with individuals in the TA flooded being wider than those in T flooded, PT flooded, PT unflooded and PTA unflooded. *Triglochin* individuals in the PTA flooded were also wider than those in the PTA flooded.

2.3.11 Number of leaves

There was no significant effect of flooding on the number of leaves for any of the species (*Aster* $F_{1,194}$ = 1.457, p = 0.229; *Plantago* $F_{1,214}$ = 0.371, p = 0.543; *Triglochin* $F_{1,211}$ = 1.812, p = 0.180). There was a significant effect of species composition in the number of leaves of both *Aster* and *Triglochin* (Aster $F_{3,194}$ = 5.233, p = 0.002; Triglochin $F_{3,211}$ = 4.248, p = 0.006), but for *Aster* the pairwise comparisons were unable to detect any significant differences. For *Triglochin maritima*, individuals in the PTA flooded had significantly more leaves than T flooded, T unflooded, PT unflooded and PTA unflooded (Figure 2.3). For the last of these, PTA flooded, there was also a significant interaction ($F_{3,211}$ = 4.991, p = 0.002). This is the same interaction as seen for height and width, with *Triglochin* individuals grown in mixed pots of all three species (PTA) being significantly shorter, narrower and having fewer leaves in the unflooded treatment.

2.3.12 Specific leaf area

Aster, *Plantago* and *Triglochin* all had significantly higher specific leaf area in the flooded treatment (*Aster* $F_{1,178}$ = 11.015, p = 0.001; *Plantago* $F_{1,189}$ = 5.911, p = 0.016; *Triglochin* $F_{1,188}$ = 4.330, p = 0.039). The specific leaf area of *Triglochin maritima* did not differ between the species composition treatment ($F_{3,188}$ = 1.677, p = 0.173). Both *Plantago maritima* and *Aster tripolium* had significantly different specific leaf area between the different species compositions treatments (*Aster* $F_{3,178}$ = 20.773, p <0.001; *Plantago* $F_{3,189}$ = 14.647, p<0.001). For both species, these responses were driven largely by a significantly larger specific leaf area in the PA unflooded treatment compare to other treatments and flooding regimes including PA unflooded (*Aster* $F_{3,178}$ = 24.997, p <0.001; *Plantago* $F_{3,189}$ = 5.386, p = 0.001; Figure 2.3).

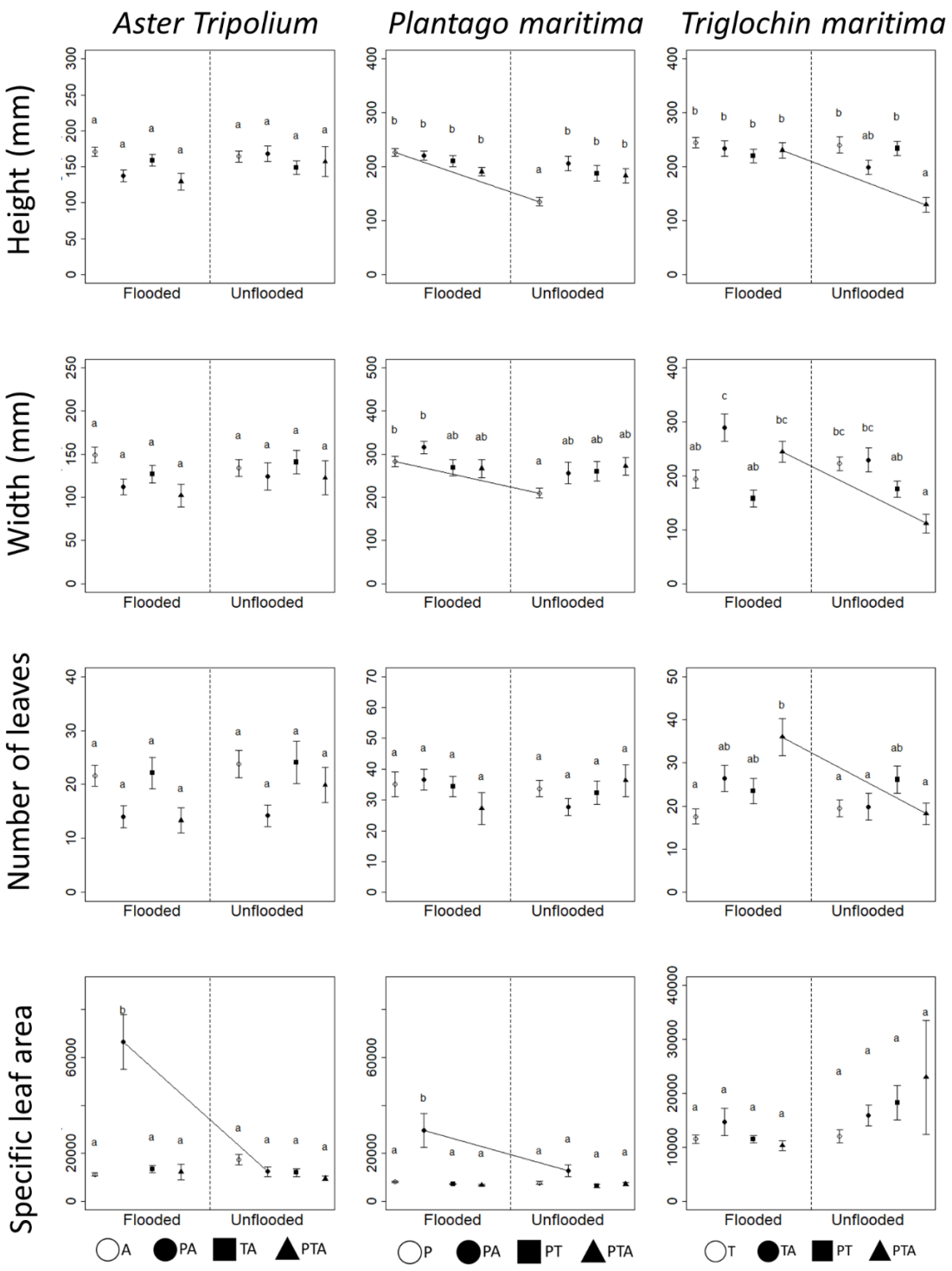

Figure 2.3 Physiological traits at species level. Columns of panel left to right, Aster tripolium, Plantago maritima and Triglochin maritima. Rows top to bottom, height (mm), width (mm), number of leaves (n) and specific leaf area (mm^2/mg). Letters in the plot refer to statistical differences derived from the Tukey post hoc test following a two-way ANOVA with interaction term. Lines between points denote significant interactions between species composition and flooding treatments. Legend key A = Aster tripolium only, P= Plantago maritima only, T= Triglochin maritima only, PA = Plantago maritima and Aster tripolium grown together, TA= Triglochin maritima and Aster tripolium grown together, PT Plantago maritima and Triglochin

maritima grown together. PTA = Plantago maritima Triglochin maritima and Aster tripolium grown together

2.3.13 Pot level analysis

2.3.14 Biomass

There was no pot level difference in total biomass or above ground biomass between flooding treatments (total biomass $F_{1,64} = 0.77$, $p = 0.381$; above ground biomass $F_{1,72} = 0.003$, $p = 0.955$). However, there was a significant effect of flooding on root mass, with pots having more root mass in the unflooded treatment ($F_{1,82} = 5.868$, $p = 0.018$). There was a significant response of total biomass, above ground biomass and root mass to the different species compositions (Total biomass $F_{6,62} = 12.0$, $p < 0.001$; above ground biomass $F_{6,72} = 6.320$, $p < 0.001$; root mass $F_{6,82} = 4.271$, $p < 0.001$). Across all three biomass measures, pots containing *Plantago* and *Triglochin* as monocultures tended to have more biomass, and the most biomass was found in pots that contained *Plantago* and *Triglochin* together (Figure 2.4). In contrast to this, pots containing *Aster* tended to have less biomass. This was not due to *Aster* being the smallest of the three species as across all three measures of biomass, mixed pots that included *Aster* were not significantly different to those with *Aster* grown as a monoculture.

2.3.15 Side-on and top-down surface area

Side-on surface area and top-down surface area both showed no response to the flooding treatment (side-on $F_{1,91} = 0.100$, $p = 0.753$; top-down $F_{1,87} = 0.211$, $p = 0.647$). There was a significant effect of species composition on both side-on and top-down

surface area (side on $F_{6,91}$ = 6.724, p <0.001; top down $F_{6,87}$ = 2.586, p = 0.024), but the response differed between the two measures. There was a general trend towards higher top-down surface area with increasing number of species and this was most apparent in the flooded treatment (Figure 2.4). However, the only significant pairwise comparisons were pots in PA flooded and PA unflooded being larger than *Aster* monoculture pots that were flooded (See Appendix 2.2 for a full list pairwise comparisons).

Side-on surface area showed a similar pattern to top-down area in the flooded treatment, with a trend towards higher side-on surface area with increasing number of species in the pots. However, this pattern was not mirrored in the unflooded treatment, with no clear pattern in the pairwise comparisons (Figure 2.4).

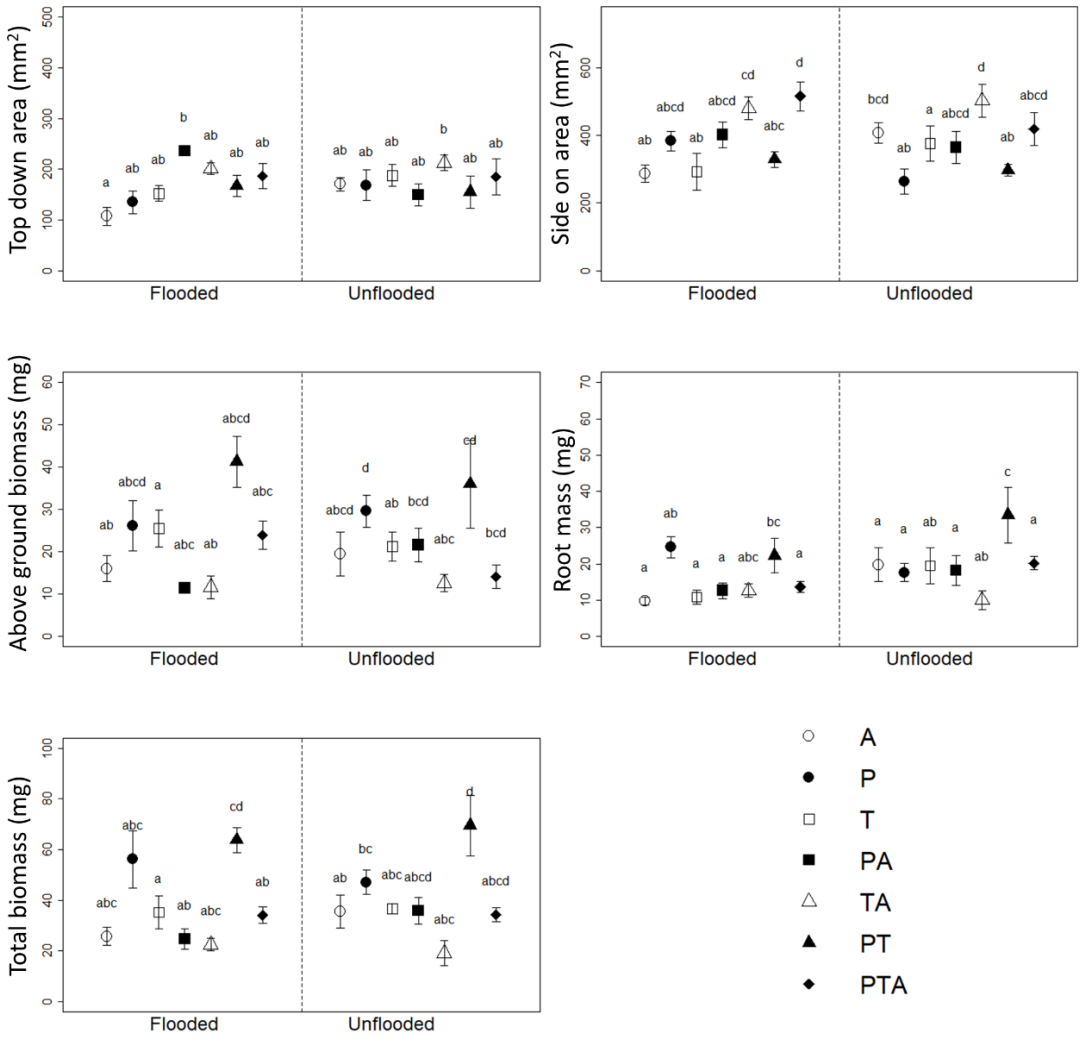

Figure 2.4 Pot level traits including all species combined. Rows left to right and top to bottom, top-down area (mm²), side-on area (mm²), above ground biomass (mg), root mass (mg) and total biomass (mg). Letters in the plot refer to statistical differences derived from the Tukey post hoc test following a two-way ANOVA with interaction term. Legend key A = Aster tripolium only, P= Plantago maritima only, T= Triglochin maritima only, PA = Plantago maritima and Aster tripolium grown together, TA= Triglochin maritima and Aster tripolium grown together, PT Plantago maritima and Triglochin maritima grown together. PTA = Plantago maritima Triglochin maritima and Aster tripolium grown together.

2.4 Discussion

Flooding alone had a relatively minor effect on the traits measured and the greatest source of variation was that of species composition. However, flooding did often have a strong influence on the interspecific interactions. Overall, we found a complex interplay between species composition and flooding effects, the strength and direction of which differed dependent on the trait and species in question. There was also substantial individual variation in traits that was not attributed to the treatments and we suggest this variation may be attributable to differences in genetic identity.

There was no consistent response in biomass across the three species to flooding. *Aster tripolium* decreased in total biomass when flooded, while the other two species showed no response; there was also limited response of biomass allocation (root:shoot ratio) to flooding. The reduction in biomass in the flooded treatment, as observed with *Aster tripolium*, is consistent with that observed in other saltmarsh species (e.g. in *Spartina alterniflora* by Visser et al., (2006)). It is therefore interesting that there was no overall effect of flooding on the biomass on *Plantago* and *Triglochin*. The lack of response by *Plantago* is surprising since in field manipulations survival is lower in waterlogged conditions (Mossman et al., 2019). In contrast, *Triglochin* is more tolerant of waterlogging. It may be that flooding conditions were not extreme enough to elicit a response (although note the response of *Aster*, which tends to occur at lower elevations of the marsh (Sullivan *et al.*, 2018)). Simas et al., (2001) predict that changes in saltmarsh productivity would only occur at the more extreme rises in predicted sea level. Since we

did not find a uniform response across all species, impacts of sea level rise may depend on the dominant species within a site.

While there was no change in total biomass for *Triglochin maritima*, flooding did result in lower root mass, suggesting a change in resource distribution. Also, while there was no significant effect of flooding on the root:shoot ratio, it was close to being significant ($p=0.068$) and there was a significant interaction between flooding and species composition on biomass allocation (root to shoot ratio) in *Triglochin*. *Triglochin maritima* has been observed to show changes in root morphology in response to flooding, growing more surface roots in flooded conditions. This response means it acts as an important ecosystem engineer, helping to raise the marsh surface and allow other species to colonise the subsequently less flooded environment (Fogel, Crain and Bertness, 2004). If an individual is devoting more energy into production of different root structures, such as the production of wide surface roots, this could result in an overall change in the total biomass of the roots. Although our study did not measure root structure, this is the first time that a change in overall biomass or a change in resource partitioning (the latter only being close to significant) has been shown in *Triglochin maritima* in relation to flooding response. As we did not measure root morphology only biomass, more research would be needed to confirm a link between changes in root structure and biomass partitioning. There was also evidence of changes in resource partitioning in *Aster tripolium. Aster tripolium* differed from *Triglochin maritima* in that the response was seen as an increase in above ground biomass. It is unclear why this may have occurred although it does suggest that different species may employ different strategies to cope with the increased stress of flooding.

In general, plants have been shown to adapt their resource partitioning in relation to different stressors (Hunt and Nicholls, 1986). Due to the number of traits measured in this study, we only targeted relatively broad measures such as height, width and biomass. It is therefore likely that we did not capture some of the fine scale adaptations, such as changes to tissue structure or ion distribution. In halophytes, we know stress can trigger specific pathways that centre on the use of organic carbon as a resource for physiological adaptation in relation to increased stress. This has typically been studied in response to salinity increases. This can involve directing growth of different tissues to compartmentalise Na^+ ions, such as in certain root tissues or towards mature leaves and away from developing leafs (Cheeseman, 1988). In the case of response to flooding stress, as in our study, it has also been shown that changes in aerenchyma tissue can also play a key role in moderating anoxic stress in halophytes (Smirnof and Crawford, 1983). It is not possible to determine whether any of these changes affected our study organisms, although the muted response to flooding observed may be because of some or all of the changes discussed above.

There has been a lot of study on the response of selected saltmarsh species, such as the invasive *Spartina alterniflora*, to a range of environmental stressors, including nutrient availability (Zhao *et al.*, 2010), salinity (Hester, Mendelssohn and McKee, 2001) and water stress (Hessini *et al.*, 2009). Whilst studies such as these provide valuable information for the species they consider, they often focus on one species or environmental gradient in isolation. This limits their impact for making inferences on functional response at the ecosystem level.

A study by Richards, Pennings and Donovan (2005) that did use a range of environmental variables found that phenotypic variation of saltmarsh plants was correlated with strong environmental gradients. They also found that this could not be explained by a single trait with the possible exception of plant height, but instead a suite of traits was needed to explain the plant response to a combination of different environmental variables. Similarly, in our study where we used a range of different species compositions instead of environmental conditions we found that no single trait best described a plants response to its neighbours or to changes in the environmental, in our case flooding pressure. Instead, we saw a range of specific situational responses the increased flooding stress, which changed based on species and was modified by its interactions with neighbouring species.

Generally, species composition had a larger influence on the biomass of the species in our study than flooding. *Plantago maritima* and *Triglochin maritima* consistently grew larger, having larger root mass and above ground biomass, when grown together. This corresponds with Silliman et al., (2015) that found mixed species plantings produced higher yields. However, this was not the case for all of our mixed plantings, with individuals of *Plantago maritima* and *Triglochin maritima* being smaller in pots containing *Aster tripolium*. Total biomass in pots containing *Aster tripolium* and either *Plantago maritima* or *Triglochin maritima* was lower than those containing monocultures of *Aster tripolium*, indicating this was not just an artefact of *Aster tripolium* plants having lower biomass than the other two species (Figure 2.2). Although the biomass of *Aster tripolium* was not influenced by species composition, this does not mean that it is not having an effect on biomass production of other species.

Interestingly, this negative effect of *Aster* was mediated by the flooding regime. Previous research has shown that the relative strength of species interactions can change along a stress gradient (Huckle et al., 2000, Crain, 2008, He and Bertness, 2014). Our results show a rather complex picture of interactions, with *Aster tripolium* negatively affecting the biomass of *Plantago maritima* and *Triglochin maritima*, particularly in the more stressful flooded conditions, and potential facilitative interactions between *Plantago maritima* and *Triglochin maritima*, which is reduced by the presence of *Aster tripolium*. There is no clear indication within our results or in the wider literature as to why *Aster tripolium* may be having a negative effect on the other two species. *Aster* is broader leafed than the other species and it may be causing shading; *Triglochin maritima* has been shown to be lose out in competition for light (Van Der Wal *et al.*, 2000). *Aster* may also be using the available nutrient resource more efficiently, e.g. it may have greater foraging precision than *Triglochin* or *Plantago* (Rajaniemi, 2011). Arbuscular mychorrhizal fungi (AMF) can alter competitive interactions in saltmarsh plant species and these effects can vary with environmental conditions (Daleo *et al.*, 2008). In field studies, *Aster* was found to have high levels of arbuscular mycorrhizal, particularly compared to *Triglochin* that had very little (Rozema *et al.*, 1986; Carvalho, Caçador and Martins-Loução, 2001), and relative AMF colonisation levels varied between species with waterlogging. We did not measure AMF colonisation in our experiment but differences in this between species may have resulted in the differences observed.

The mix of positive and negative interspecific interactions we found, has also been observed in other related species (Bruno *et al.*, 2017). Bruno et al. (2017) found a multitude of positive and negative interactions of *Aster tenuifolius* with other saltmarsh

species and that these interactions changed over the life cycle of the individual. We only observed interactions in young adult plants and further studies at different life stages would be beneficial, particularly since species interactions are important in shaping the species assemblages (Callaway et al., 2000).

Previous research has shown that greater biodiversity leads to an overall increases in biomass of saltmarsh (Callaway, Sullivan and Zedler, 2003) and other ecosystems (e.g. Cardinale et al., 2007). We also know that changes in a single species can have large effects on biomass or functioning (Symstad *et al.*, 1998). Our results agree with this research, showing the presence of species interactions increase biomass above that of a monoculture, as well as the presence or absence of a single species (*Aster tripolium*) were sufficient to change the biomass of the entire pot. Our use of only three species reduces our ability to extrapolate to marsh-level effects. However, local communities of three to four species are common on saltmarshes (Mossman et al., 2012) and using three species allowed us to distinguish the interactions between all species combinations, which a larger pool of species would make less practical.

Although total biomass of *Plantago maritima* and *Triglochin maritima* was not influenced by flooding, there was a response in their structural morphology. *Plantago maritima* and *Triglochin maritima* individuals were taller, wider and had more leaves in the flooded treatment; *Aster tripolium* showed no response in height, width or number of leaves to flooding. This is very similar to the response of another saltmarsh species, *Juncus kraussii,* that has been found to grow larger under flooded conditions (Naidoo

and Kift, 2006). The same study also altered salinity and found that it was the combined effect of low salinity and flooding that led to the larger growth of *Juncus krausii* (Naidoo and Kift, 2006). Whilst we did not alter salinity in our experiment, we did use 50% rather than 100% sea water due to concerns of evaporation inside the glasshouse. We therefore replicated very similar conditions to those used in Naidoo and Kift, (2006) and found the same result for two other saltmarsh species, suggesting this phenomenon may be widespread amongst halophytes. Halophytes such as *Plantago maritima* and *Triglochin maritima* are well adapted to stressful conditions and have many mechanisms to adapt to changes in conditions (Nilsen and Orcutt, 1996). Saltmarsh plants have also been observed to show high level of plasticity in response to changing conditions (Jefferies, Davy and Rudmik, 1979). One or more of these adaptations is likely to be playing a part in allowing the species to adapt their structural morphology to the changing conditions whilst still maintaining the same levels of biomass.

In our experiment we found several examples of trait responses to interspecific interactions being heavily modified by the presence of inundation stress. For *Plantago maritima* and *Triglochin maritima,* we found a response in height, width and number of leaves in the species composition treatments. These responses were also mediated by the effects of flooding. *Triglochin maritima* grown with both other species was wider, taller and with more leaves in the flooded treatment than in the unflooded, and this is the opposite of the interaction for root mass in the same species combination. Although we did find an effect in above-ground biomass, the reversal of this interaction from root mass may also be a result of changes in resource partitioning. The other interaction was the strong response of specific leaf area in mixed pots of *Plantago maritima* and

Aster tripolium for both species in the flooded treatment. Specific leaf area has been shown to change as a competitive response for light acquisition (Cannell and Grace, 1993), and it has been highlighted as one of the key traits that can infer a competitive advantages (Grotkopp and Rejmánek, 2007).

Side-on and top-down area both show a response to species composition (but not flooding), with an overall trend for an increase in density with more species. While our conclusions are limited by our use of only three species, we did find similar results to that of Möller, (2006) which found higher levels of diversity led to higher levels of vegetation density in saltmarsh. Their study was designed to investigate how species diversity of saltmarsh influences vegetation density and the subsequent effects on wave attenuation properties. This is important as we found that even a small increase of one or two species in place of a monoculture can have important impacts on ecosystem functioning. This adds to a growing body of research that increased diversity of saltmarsh can lead to an increase in ecosystem functioning, with studies such as Ford et al., (2016) also finding that higher diversity is also linked to greater soil stability in saltmarsh.

We found no effect of flooding or species interactions on survival and across all species, the survival rate was over 97%. As we aimed to measure functional traits, the high survival was consistent with our aim of changing conditions with the range of environmental tolerances of the species, e.g. those described in Sullivan et al., (2018). If

we had the experiment continued for longer, we would have expected to see an eventual effect of species competition on survival because some of the negative interspecific interactions uncovered by our measurements of functional traits would likely increase in intensity as plants increased in size.

There were very few reproductive structures produced by our species during the experiment, with only five and two individuals of *Aster tripolium* and *Triglochin maritima*, respectively, producing structures. *Triglochin maritima* rarely produces any reproductive structures in the first year of growth, even within a heated glasshouse (Davy and Bishop, 1991). The lack of reproductive structures in the relatively short-lived *Aster tripolium* is more surprising. *Plantago maritima* produced more reproductive structures than the other two species (n = 22). More rapid production by one species could have a large influence on marsh colonisation via the founder effect (Grime, 1998). We know that species community in a developing saltmarsh, such as that of a newly created managed re-alignment, can be heavily influenced by seed availability (Rand, 2001). Wolters et al., (2008) demonstrated that this can lead to long lasting effects by showing that a very common and widespread species, *Puccinellia maritima,* was still excluded from parts of its niche by other earlier colonising species, even after five years of colonisation.

Previous research has shown that *Plantago* can adapt its reproductive strategies according to environmental conditions. For example, populations of *Plantago maritima* in less flooded and less grazed areas have been observed to be short lived, reproducing

mainly by seed, and lower-lying, heavily grazed populations being longer lived and reproducing primarily via vegetative growth (Blom, 1983). While we did not find any response in reproduction to changes in environment (flooding in our study), we did find that there was a significant effect of species composition. *Plantago maritima* plants grown with *Aster tripolium* had more individuals with reproductive structures present. This may be a competitive response in relation to the presence of *Aster tripolium*. However, it could also have been a result of facilitation with *Plantago maritima* benefiting from the presence of *Aster tripolium* and hence being better able to cope with the conditions and put more energy into reproductive structures.

As well as the variation between the different treatments, there was also a large amount of intraspecific variation within a treatment (see Appendix 2.3). A likely cause for this is differences is the genetic makeup of the individuals used in the experiment. Seeds for all species were sourced from several different geographic regions, as well as from across a range different environmental conditions within the collection sites. This was to ensure there was no bias towards a specific genetic identity, so that results would be applicable regardless of population or genetic identity. This sampling regime is likely to have introduced considerable variation in genetic identity, which can impact plasticity in response to environmental conditions (Richards, Pennings and Donovan, 2005). Knowledge of the role of genetics in saltmarsh ecosystems is currently limited. We know that across the UK different geographic areas host distinguishable genetic populations of some saltmarsh species (Rouger and Jump, 2014) and that within an area, genetic diversity can be structured across environmental gradients (Rouger and Jump, 2015 , Foust et al., 2016). Our results would suggest that the sample acquisition method did

induce considerable genetic variation and that this accounted for the range in traits observed within a species. The original aim of our seed sampling design was to include as much genetic variation as possible and along with our results we can be reasonably confident that genetic variation did play a role in individual functional trait response. More detailed and more targeted studies into the role of genetics in the formation and functioning of saltmarsh would appear to be an exciting and potentially fruitful avenue of research for better understanding of these important habitats.

At first the results from our study would appear to show that increases in flooding, such as those likely to be induced by predicted sea level rise over the next decade, dependent on local sediment dynamics, would have limited impact on the functioning of saltmarsh species. This could infer a certain level of resilience and lead policy makers to focus restoration and management strategies on improving other factors such as biodiversity, which our study showed to have a net positive effect on functioning. However, our study highlights that changes in flooding could heavily modify the interspecific interactions that underpin the net positive effects of increased diversity. Therefore, we recommend a greater emphasises is put on examining the intricacies of species interactions in future research so we can fully understand the potential impact of environmental change.

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Chapter 3: Development of a self-contained tidal inundation machine and nutrient filtering system

3.0 Abstract

Coastal eutrophication and sea level rise are two of the biggest threats currently facing saltmarsh ecosystems. Studying their effects in the field is extremely difficult requiring long term, large-scale experiments. Despite this difficulty, they remain the most appropriate method as laboratory studies do not currently replicate true tidal cycles and thus the nuances associated with their influence. Previous attempts to build systems that accurately replicate tidal inundation in a laboratory setting have been over complicated or impractical for most experimental designs. Miller and Long, (2015) describe one of the best designs for a machine that can replicate tidal inundations simply and efficiently however their system has some major drawbacks. The open design of the system means that it requires a constant supply of water, it also makes no allowances for the control of water chemistry such as nutrient concentrations. We have designed and tested a new system based on Miller and Long's, (2015) earlier design that utilises a recirculating water supply and incorporates an interchangeable filter system. We tested our systems ability to replicate real life tidal cycles and also the ability of the filter system to strip nutrients. The system was able to faithfully replicate a real-life tidal regime and remove nutrients far in excess of those used in previous nutrient addition studies. This opens up new research opportunities to study the combined effects of nutrient additions and sea level rise in a controlled setting as well as providing a flexible platform for other coastal researchers to adapt to their own research needs.

3.1 Introduction

Excess nutrient enrichment in terrestrial systems and eutrophication in aquatic environments are among the most significant global environmental problems (Willem et al., 2013). Human activities and technological advancements are producing nutrients, such as nitrates and phosphates, at a rate that far out strips their production in natural systems (Rabalais et al., 2009). Human production of these nutrients continues to rise, affecting every major biological system on earth (Rieuwerts, 2016). Levels of mobile nitrogen look set to increase in the atmosphere, the world's oceans, soils and groundwater (Vries et al., 2016). The deleterious effects of this, such as habitat degradation, loss of water quality and decreases in biodiversity, have been well documented in a range of systems (Smith, 2003; Bobbink et al., 2010).

The mobile forms of nitrogen that make up the bulk of anthropogenic nutrient enrichments has led to increased nutrient loads in aquatic systems. The size and scale of the problem is increasing, with some areas experiencing a 15-fold increase in nitrate levels within a few decades (Howarth, 2008) and in the USA alone, 67% of coastline ecosystems have symptoms of eutrophication (Bricker et al., 2008). Saltmarsh is one of the most important coastal ecosystems and we have strong evidence that they are extremely vulnerable to the effects of increased nutrient loads. For example, an experiment by (Deegan et al., 2012) showed that increased nutrient loads caused a decrease in below ground biomass and increase in microbial decomposition of organic matter, ultimately leading to a critical failure of the creek banks and complete erosion of saltmarsh.

In addition to nutrient enrichment of coastal waters, increasing inundation as a result of rising sea levels (Crosby et al., 2016) is causing further increases in nutrients, and is a major threat to the survival of these environments (Gan, 2014). We know that nutrient exchange in these systems is dictated by the tidal cycle (Whiting et al., 1989) and this tidal cycle will change as sea levels rise (Bamber et al., 2019). Current estimates predict a rise of between 0.7 m-1.2 m by 2300, even if we stopped greenhouse gas emissions immediately (Mengel et al., 2018). Saltmarshes are particularly vulnerable to the effect of sea level rise as they are often blocked inland by retaining sea walls. As sea levels rise, the area seaward of the sea wall suffers increasing frequency and duration of tidal elevation and the lowest lying areas are gradually lost to the sea, a process known as coastal squeeze (Torio and Chmura, 2013). Of the areas of saltmarsh that remain, changes in flooding regime have been proven to alter plant communities. For example, species that usually inhabit lower elevations such as *Spartina alterniflora migrating further up the marsh (Donnelly and Bertness, 2001). Sea level rise is also predicted to cause a change in the ecosystem service delivery of saltmarsh due to changes in species distribution and microbial communities (Craft et al., 2009).*

One method of studying the effects of increased nutrient levels and sea level rise is via large-scale field experiments, such as those carried out by Deegan, (2002) and Johnson et al., (2016). These studies have enriched large areas (60,000 m^2) over the course of a number of years (9 yrs) Johnson et al., (2016). These studies offer the best way of looking at the effects of nutrient concentrations in real environments, but they have some

significant drawbacks. Firstly, we know that there are negative effects of increased nutrient concentrations and so large-scale experiments on important ecological sites risk damaging the environments we wish to protect. Secondly, with the widespread nutrient enrichment that already exists, it is difficult, if not impossible, to have true controls in such experiments. This is particularly difficult in aquatic systems due to the higher mobility of nutrients compared to terrestrial systems (Elser et al., 2007). This high mobility also makes it difficult to control for external influences of other nutrient sources on the experiment, making it hard to separate out the finer details of the study, such as the effects of different nutrient sources. It is also impossible to replicate the predicted levels of sea level rise in large-scale field experiments without considerable alteration to the environment, which in turn risks introducing more variables into the study. Studying the effect of sea level rise in the field usually requires modification to elevation and even fine changes in topography risk changing environmental variables, such as flooding regime and redox potential (Mossman et al., 2019). Finally, large-scale field studies are often prohibitively expensive requiring large amounts of nutrients and effort to complete.

At the other end of the scale are smaller glasshouse experiments, such as Hanson et al., (2016) but these also have their limitations. Their relatively small scale constrains the ability to replicate the number of a variables present in a natural system. More importantly there are also some variables that are difficult to replicate in a laboratory setting, such as the tidal regime. The tide affects many different things within coastal sites such as hydrological regimes, sediment influxes and erosion, and herbivory as well as changes in the levels and the mobility of nutrients within a system (Whiting et al.,

1987; Asmus et al., 1998; Lynn and Reed, 2018). Due to the inherent complexity of replicating a true dynamic tidal cycle, experiments often use simpler measures to replicate them. This usually takes the form of a binary variable of inundated or not inundated (for example Szura et al., 2017; Sun et al., 2018 and Wang et al., 2019). A flooded-unflooded variable fails to replicate the complexity of a full tidal regime. Unlike a binary flooded-unflooded variable, a natural tidal cycle changes over days and months causing changes in the number and frequency of inundations over time. The frequency and duration of inundation has been shown to change across elevation gradients on saltmarsh (Eleuterius and Eleuterius, 1979) and has been directly linked to the formation of distinct vegetation zones (Bockelmann et al., 2002). It is vital to understand how changes in inundation regime will effect saltmarsh, as increased inundation is a proven stressor to plants (Engels et al., 2011). In addition, differential tidal inundation frequency leads to changes in soil chemistry, affecting the redox potential of the soil as well as the movement of nutrients through the site (Armstrong et al., 1985; Gao et al., 2018). Without a system that can adequately replicate this tidal inundation we are not able to study the true effect of graduated tidal inundation or to untangle the relative impacts of inundation and nutrient stressors in a controlled laboratory setting.

This study aims to design a more practical system for accurate replication of tidal inundation and nutrient control in a laboratory setting. In order to do this we describe a system developed to create real time tidal inundations using an enclosed and filtered water supply, for simple accurate and precise control of water chemistry. We first develop a tidal inundation machine (TIM) utilising a recirculating filtered water supply and then test for the maximum nutrient removal capacity of the filtration system within

normal operating parameters. We test the filtration system by running a 20-day real time tide table with increasing nutrient concentrations to establish the maximum removal capacity of the system within the space of one tidal cycle. The system was set up to allow half of the experimental tanks to remain nutrient poor and the other to become increasingly nutrient rich, with all water being returned to a single municipal reservoir between inundations.

3.2 Methodology

3.2.1 Summary of system design

The system consists of a 1000 litre sump tank connected to a tidal control rack as described in (Miller and Long, 2015), feeding two sets of two connected experimental tanks. The tidal rack is controlled by an Arduino mini ©, which raises and lowers the rack in time with a pre-programmed tidal cycle. The raising and lowering of the rack causes the water in the experimental tanks to rise and fall in line with the true-to-life tide cycle. The parameters for the tide are controlled by the Arduino computer and the code allows replication of the tidal cycle at any location for which there is information on the requisite tidal harmonics. At low or falling tide, water is returned to the main sump via a smaller sump and pump. At high tide, water is fed into the main tanks via gravity and the maximum level is dictated by the height of the rack and therefore connected pipe. The water in the main sump is continually filtered to remove excess nutrients in the system, allowing the two sets of experimental tanks to be consistently dosed with

differing nutrient concentrations. All this can be achieved without the need for a constant supply of saltwater and in minimal space with only the need for one large sump and filtration system unlike the open design of Miller and Long, (2015). Figures 3.1 and 3.2 provide visual overviews of the design of the tidal inundation machine, which is described in more detail below.

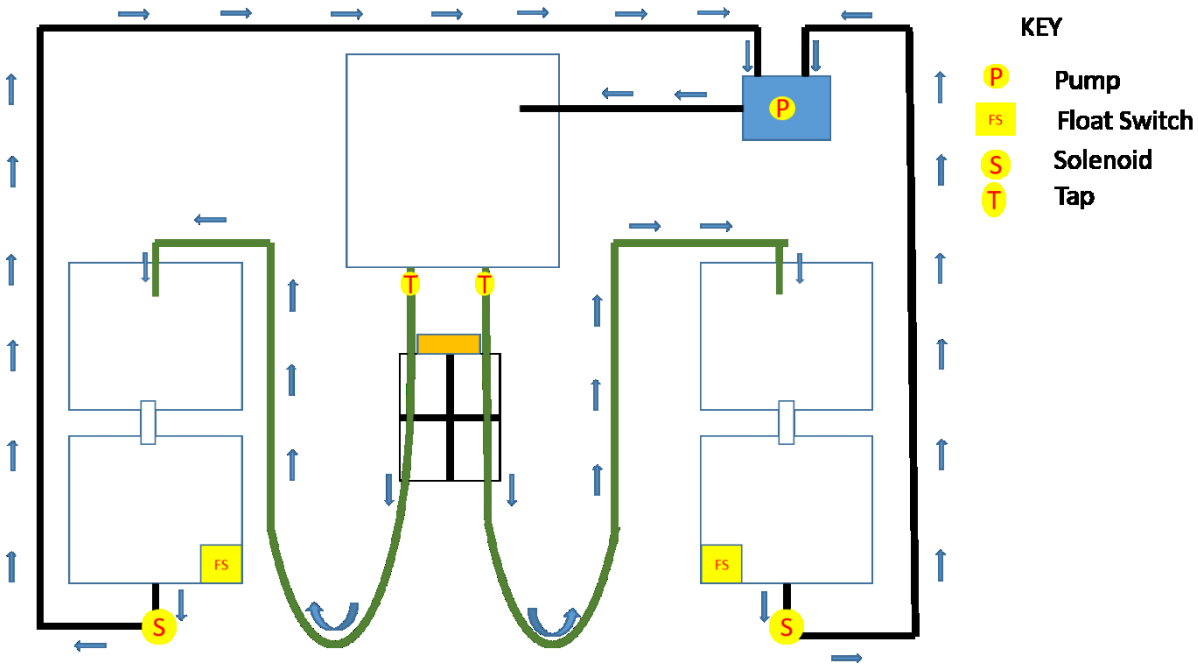

Figure 3.1 Functional diagram of tidal inundation machine. Blue arrows show direction of water movement through the system

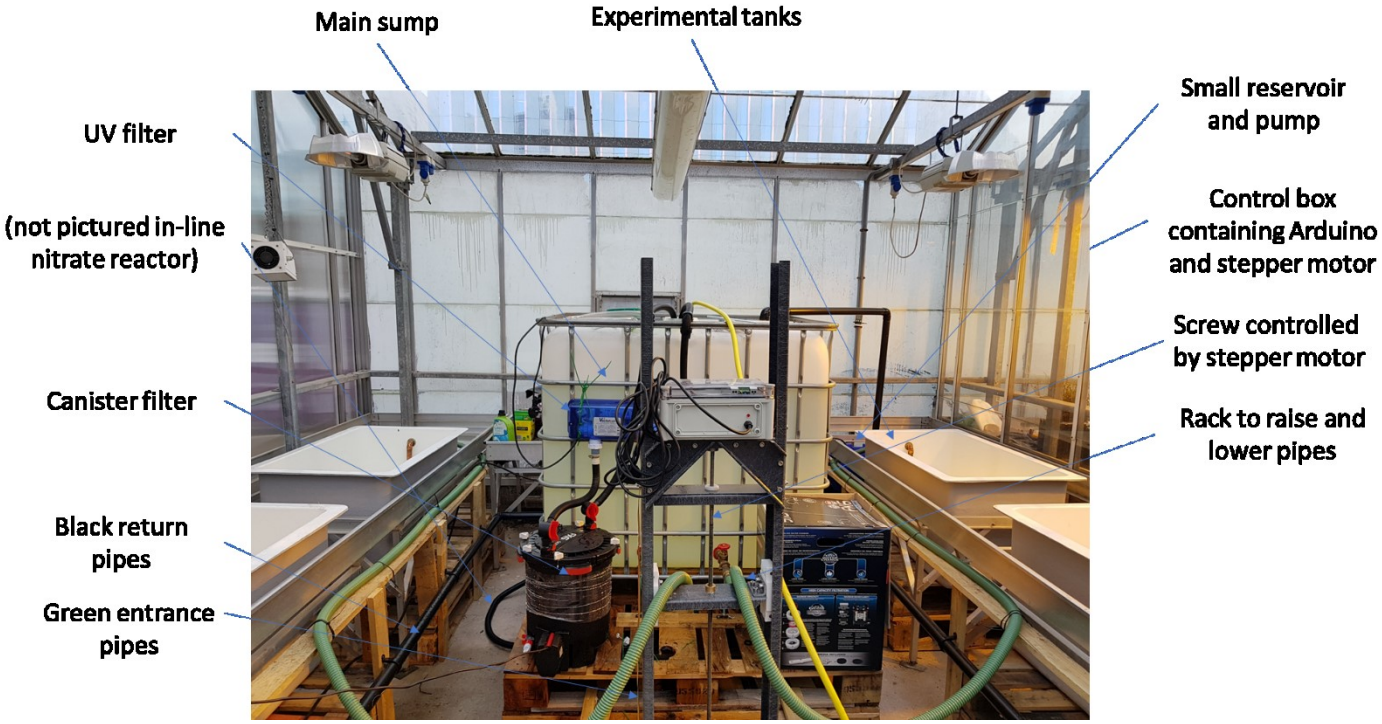

Figure 3.2 annotated picture of Tidal Inundation machine showing key components

3.2.2 System construction

The sump tank is comprised of a commonly available square 1000 litre intermediate container (ICB) tank. The tank has been modified from a standard ICB tank to include two pipe connections at the bottom of the tank, as well as an inlet on the roof of the tank for returning water. Attached to the two outlet pipes at the bottom of the tank are two-compression isolation taps that allow the water in the sump to be isolated for maintenance and to adjust the flow of water during regular operation. The two outlet pipes are large and flexible to minimise the effects of drag, reducing any lag in the change of the tidal height in the systems. The two pipes carrying the water from the sump pass through the tidal control rack (detailed below) and into one of the two sets of joined experimental tanks. At the outlet from the sump tank and the inlet of the experimental tanks, the pipes pass through an open topped T valve to prevent any effect of siphoning.

The main design concept of the tidal control rack remains unchanged from Miller and Long, (2015), which contains the full technical specifications of their design. The rack consists of a fiberglass frame supporting a large screw and a plateau on which pipes can be mounted. The screw is controlled by a stepper motor, which turns the screw raising and lowering the plateau and attached pipes. The stepper motor is connected to an Arduino microcontroller, which via use of an embedded code raises and lowers the screw in line with a pre-determined tide cycle. There is also an internal clock attached to the Arduino controller to allow the system to keep track of time, raising and lowering the tide accordingly. The rack designed for our system uses metric measurements and

is 30 cm taller than that described in Miller and Long, (2015) to accommodate the height of our experimental tanks. We have also included a more powerful stepper motor to accommodate for the larger size and weight of the screw and pipes used in our set up. We have made numerous changes to the code for the Arduino micro controller to ensure compatibility with our system, as well as to omit the use of an led panel, as this was not included in our set up.

Each set of experimental tanks consists of two 750 litre tanks linked by a large diameter rigid PVC pipe located at the bottom of the tanks. Each set of tanks can be dosed with different nutrient loads separately prior to water being returned to the main sump, where the water is then filtered and nutrients removed before the next tide, allowing for two separate experimental conditions. Additional tanks can be added in series to each set to increase the experimental replication or space. Additional sets of tanks can also be added to allow additional experimental treatments, and is only dictated by the size of the rack power of the motor, and the volume contained in the main sump tank relative to the size of the experimental tanks.

Inside each tank is a ball and float switch connected to a solenoid valve at the opposite end of the tank from the water inlet from the sump tank. The ball and float switch has an adjustable float and is set to trigger at a height just below the lowest high tide programmed for the duration of the individual experiment. Once activated the float switch triggers the solenoid valves, which open allowing water to drain out of the experimental tanks. The outlet for the water is a rigid PVC pipe set on an incline

depositing water into a small 200-litre reservoir. This reservoir contains another float switch that triggers a submersible pump once the reservoir has reached capacity. The pump then returns all the water in the reservoir back to the main sump completing the cycle.

The incline and diameter of the pipes returning water from the experimental tanks to the main ICB tank are set so that the maximum return flow is always lower than the minimum flow of water into the experimental tanks on a rising tide. This ensures that when the float switches trigger, the level of water in the experimental tanks remains constant until the tide drops below the level of the float switches. Whilst theoretically the float switch and solenoid combination could be left out of the system, this would cause continuous recirculation of the water at high tide so that the water would not have chance to become adequately filtered. Depending on the amount of water in the system, the pressure in the main sump has the potential to overcome the gravitational resistance of the pipes causing the experimental tanks to fill even at low tide. Regulation of this pressure is achieved via adjustable valves on the main sump to allow for fine scale adjustment of flows. The intermediate reservoir must also be large enough to accommodate the water passing through the system between the time the solenoid valves are opened and the time the tide level starts to fall to avoid contamination of the filtered water in the main sump with the water that has already passed through the experimental tanks. In our system a 200 litre tank was more than adequate for this purpose.

The original system for generating tide data for the tide controller required three steps. Tidal harmonics to be generated by the open source program “x tide” (Flater, 2005). This data was then copied into an R script to be ran in the open source software R studio (R Studio Team, 2019), which creates a library of the tidal harmonics in a readable format. Finally, the harmonics database was fed into an R script, which creates usable tide height data over time to feed into the Arduino script. This system, detailed in the supplementary material of Miller and Long, (2015), either required a National Oceanic and Atmospheric Administration (NOAA) tide station ID number from which to acquire the tidal harmonic data from NOAA’s open database, or manual generation of the tide data from the requisite tidal harmonics. We adapted the code for using packaged tidal harmonics for NOAA to instead accept data from our own database of tidal harmonics from the UK and Europe.

3.2.3 Filtration system

The filtration system effectively runs a separate closed water recirculation system to that of the main sump and experimental tanks, thus does not interfere with tidal replication. This means that it can easily be replaced with a different filtration method appropriate for different experimental designs without influencing the tidal replication. We used a canister filter due to it being the most efficient option given the space we had available. A cheaper and potentially more efficient option would have been to install a separate sump filtration system. Such as system would also need a separate closed nitrogen reactor if you wished to filter nitrates, as we did in our test experiment, as these will not function in the aerobic conditions of an open sump.

These filters were added to the system to remove nutrients, allowing addition of nutrients to a subset of experimental tanks, and prevent build-up of free-floating algae, common in closed water systems. The nutrients are removed via chemical filtration (phosphates), biological filtration by denitrifying bacteria (nitrates) and the algae is removed by a UV steriliser. The system consists of three parts, a Fluval xf6 canister filter, an inline nitrate reactor (Aquirapore) and Fish lab 1000 UV filter. The Fluval fx6 is placed below the sump at floor level to allow for siphoning of the main sump and water is returned via the inline nitrate reactor by the embedded pump. The return pipe of the nitrate reactor is connected to the UV filter before exiting back to the main sump. The inlet is positioned in the bottom of the sump and the outlet is attached to a spray bar at the top of the sump above the water line to allow for adequate mixing and also aeration, increasing the efficiency of the filtration process. The Fluval fx6 is self-priming, automatically creating a syphon effect when turned on and has an auto start and stop function inbuilt for low water levels. This allows the filter to stop and start automatically when the sump empties below operating limits.

The Fluval fx6 is a modular canister filter, with water passing through a series of baskets containing filtration media, and thus filtration media can be easily swapped out to match the needs of different experiments. In our set up, we used the stock sponge and fluvial ceramic beads for mechanical and biological filtration. The sponge traps particulate matter, whilst the sponge and ceramic beads provide a high surface area to foster colonisation of denitrifying bacteria. The use of denitrifying bacteria means the system

requires an initial growth phase where nutrients are constantly added in small quantities building up to the desired level for the experimental operating parameters. Once established, the bacteria require a constant input of nutrients to maintain the colony and thus removal rates. The same process is also required to prime the inline nitrate reactor. The UV filter used was a 20-watt, 50 cm length, 0.5mm glass depth UV filter with a flow rate set to 800 litres per hour. Its purpose was to remove free-floating algae from the system, a common problem in high light and nutrient environments. Dependent on experimental setting, a UV filter may not be necessary. Our system was installed in a glasshouse and intended for use in high nutrient plant growth experiments and thus required the use of UV sterilisation to maintain water quality.

3.2.4 Test of accurate tidal replication

In order to test that the tidal inundation machine was accurately replicating desired tidal cycles, we firstly compared the tidal cycle generated by the on-board micro controller to historic data for a local tide station. To do this we calculated the tidal data for Liverpool Gladstone dock in August 2016, and extracted high and low tide heights and times predicted by our micro controller and compared these to historic data for the same tide gauge and in the same time period, sourced from the British Oceanographic Data Centre (BODC) (BODC, 2016). We then ran this tide in real time in our experimental set up and periodically tested the levels in the system at the predicted high and low tide times using a manual float measure. For this test, we calibrated the machine so that the bottom of the tanks would simulate an elevation of 1.9 m above sea level, as this was the mean low tide height for the month. This gave us the greatest opportunities to

observe variation in tidal levels across the month and ensure they were being accurately replicated in the system. For practical purposes, we only tested water levels of high and low tides that fell during normal opening hours, between 07:30 and 18:00 during the month of testing.

3.2.5 Nutrient removal test

To test the TIM's ability to simulate real world inundation and control elevated nutrient loads, we ran an experiment to test the maximum capacity of our filtration system to cope with phosphorous and nitrogen inputs. The system was running a tide for Liverpool Gladstone dock between 9th & 29th April 2018. One set of experimental tanks was dosed with nutrients and the other was left untreated. We measured the nutrients in the sump at low tide each day.

Prior to the experiment, the sump tank was filled with a combination of tap water and marine salt (Instant Ocean® Blacksburg, Virginia) to replicate a solution of 50% strength seawater (specific gravity 1.013 at 25˚C). Marine salt was used as we did not have access to fresh seawater and this has been proven to be the most appropriate alternative for testing the effects of sea water on coastal plants (Hanley et al., 2019). The filter set up, excluding the inline nitrate reactor (Aquirapore), was allowed to run on the water in the sump tank uninterrupted for 12 weeks whilst being dosed with ammonia to establish the bacterial colony necessary. The nitrate reactor was only introduced in the last two weeks once nitrates had started to appear in the system. This is the same process that

is used to establish denitrifying bacteria in home aquariums. Sponge from a filter running on a fully cycled, long established home aquarium was used in the experimental filter to help seed the initial bacterial colony. Similarly, the denitrifying unit was taken from an established marine aquarium set up. Using pre-established media is not necessary but helps to reduce time for establishment and decreases the risk of failure. For the experiment detailed below, we used the stock sponge and 500 grams of Fluval ceramic beads to form the cultivation surface for the bacterial filtration. We placed 750 grams of active carbon in the top compartment of the filter; this was used as a generic all-purpose chemical filter as it binds well with many toxic metals and organic particles (DeSilva, 2000). The second of the three compartments housed 500 grams of Phosguard (Seachem© Madison, Georgia), a silica compound formulated to absorb phosphates from the water. The third compartment housed the ceramic beads. Both the Phosguard beads and activated carbon would need replacing periodically once their carrying capacity had been exhausted.

One set of tanks was dosed at low tide with 500 ml of a solution of dissolved Ammonium Nitrate and Sodium Phosphate (ratio N:P 4:1) to achieve the desired concentration of nutrients once the tanks were full of water at high tide (experimental concentrations described below). The ratio used in our experiment was lower than the 16:1 commonly found in coastal waters (Downing, 1997) and used for nutrient addition experiments in large-scale field studies (Deegan et al., 2012). This ratio was used as filtration of phosphates requires a much smaller filtration set up then filtration of nitrates. As we were using a combined canister filter with equal sized compartments for filtration media is was anticipated that we would exhaust the maximum nitrate removal potential well

before phosphate if we used a higher ratio. The other set of tanks was left un-dosed. After dosing, the tide cycle was allowed to run and water was returned to the main sump on the following low tide. We then measured the nutrient levels in the sump before the next tidal cycle began (minimum 6 hours).

In order to establish the maximum capacity of the filtration apparatus, nutrients were dosed in increasing amounts from 1-60 ppm of nitrogen and 0.5 -15 ppm of phosphate. As the filtration apparatus works solely on the main sump tank and we measured the concentrations in the sump tank, we express nutrient concentrations and removal capacity as experienced in the 1000 litre sump tank. As we only dosed one half of the experimental tanks, the actual values experienced within these tanks would be approximately double that of those in the main sump tank. Nutrient dosing was conducted every day, unless the concentrations of either nitrate of phosphate did not drop below 5 ppm, in which case the tanks were not dosed and the concentration in the sump tank re-measured after 24 hours. The design and size of our initial filtration set up was chosen to filter levels of nutrients above 35 ppm, as this was the level that has previously been shown to be deleterious to wetlands in field studies (Deegan et al., 2012). In order to thoroughly test that we were meeting this requirement, we repeated the dose at 35 ppm three times to ensure compatibility at this level for future experiments (Chapter 4), before increasing the dose to find the maximum removal capacity. The experiment was ended when two attempts of the same concentration failed to be removed from the system within 24 hours. Water parameters were tested using the JBL Aquarium test lab, a commercially available set of chromatic tests for ammonia, nitrites and nitrates, and phosphates.

3.3 Results

3.3.1 Tidal Replication

The simulated tide generated by the on-board micro controller for Liverpool Gladstone dock in August 2016 was never more than 1 cm different to the historic reference data (Figure 3.1), indicating that my code replicated the predicted tides correctly. A more detailed view of the first 10 days of this comparison can be seen in Figure 3.2 to more clearly visualise the data.

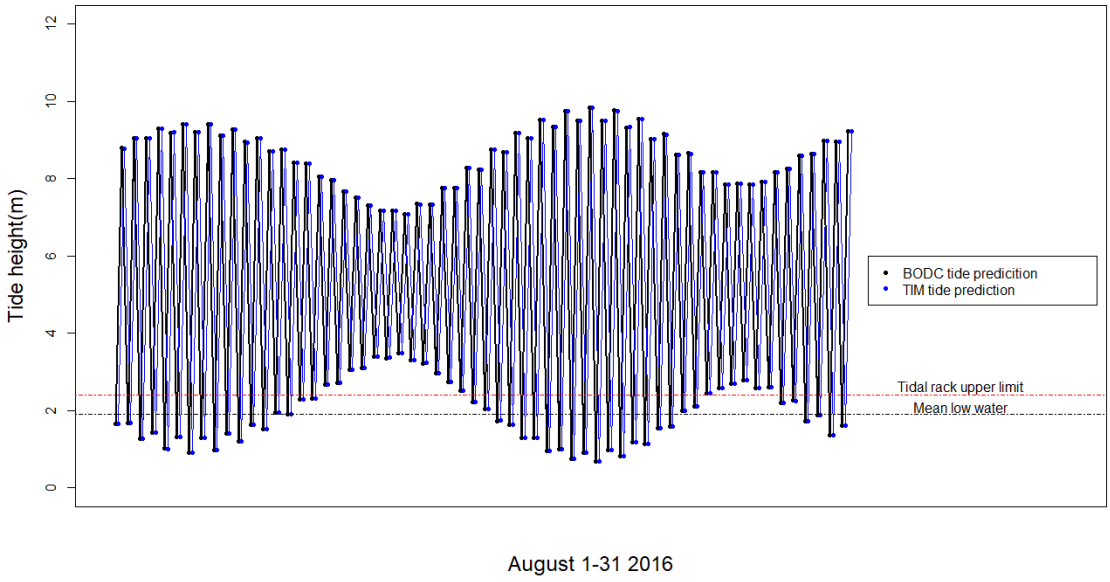

Figure 3.1 Comparison of the tidal regime as generated by the new tidal inundation machine and historically generated data sourced from the BODC for Liverpool Gladstone dock August 2016. Mean low water level is illustrated as this is set as the lower travel limit for the tidal inundation machine during testing. Similarly, tidal rack upper limit is illustrated as this was the maximum upper travel limit of the rack within the tidal inundation machine.

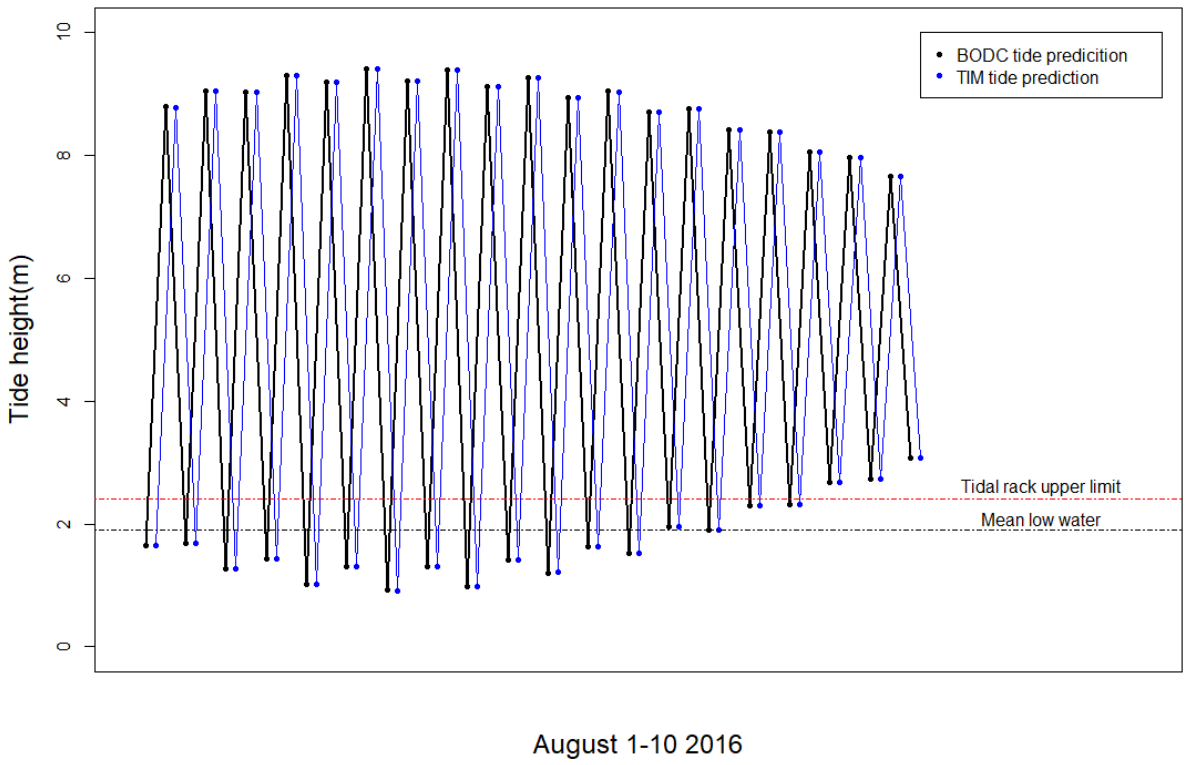

Figure 3.2 Comparison of the tidal regime as generated by the new tidal inundation machine and historically generated data sourced from the BODC for Liverpool Gladstone dock first ten days of August 2016. Mean low water level is illustrated as this is set as the lower travel limit for the tidal inundation machine during testing. Similarly, tidal rack upper limit is illustrated as this was the maximum upper travel limit of the rack within the tidal inundation machine.

As seen in Figure 3.3, all of the 44 tidal heights observed in the tanks were never more than 1 cm different from the expected value based on the tidal calculation at the time of observation. In addition, when the tidal prediction was below mean low tide the tanks were empty as expected, and when the tidal prediction was above the maximum height of the rack, the tanks were full of water to their maximum capacity.

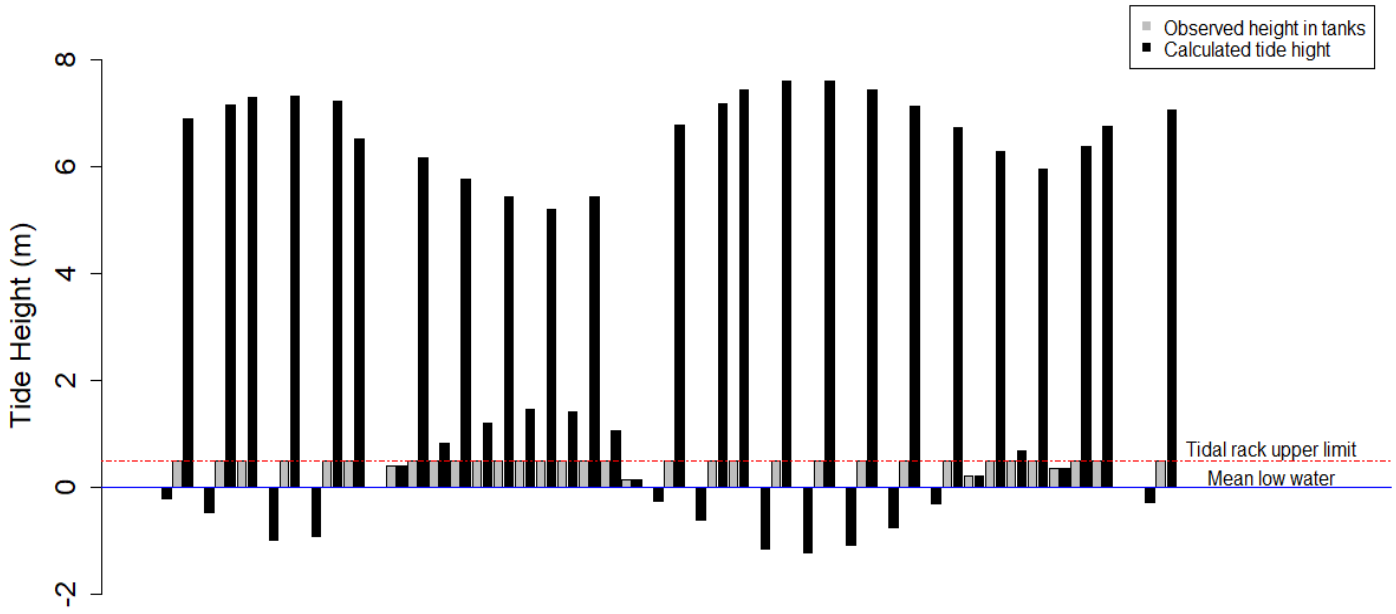

Figure 3.3 Water levels observed in the tidal inundation machine compared to the calculated tide heights for Liverpool Gladstone dock August 2016. Mean low water is illustrated as the lower limit of the tidal rack was calibrated to this value meaning it would not reproduce any tide below this level. The upper limit of the rack is also illustrated as this was the maximum height the machine could simulate. For tides above this height the water level and travel of the rack where maintained at their highest point until the calculations for tidal height dropped below this value.

3.3.2 Nitrate

The filtration system was able to reduce nitrate levels in the water to below detectable levels within the space of one tidal cycle, up to a concentration of 50 ppm in 1000 litres of water. However, there were two instances, one on day 8 with a dose of 35ppm and one on day 12 at 50 ppm, where nitrate levels were not reduced to below detectable levels within the space of one tidal cycle (Figure 3.4), although complete filtration at these levels were achieved after a repeated dose on day 9 and 14. The failure of the first dose and success of the second repeat dose in both instances can be attributed to the lag in bacterial colony growth of the biological filter in response to the increase in nutrient concentration. At 60 ppm, we were unable to remove all nitrates successfully within the space of one tidal cycle (Fig. 3.4). We repeated this concentration twice before stopping the experiment. The first dose at 60 ppm took three tidal cycles to be fully cleared and the second took eight. The increasing time for filtration levels was an indication that we had reached capacity of the biological filtration media with bacteria population numbers having expanded beyond sustainable levels and crashing. This is a process where the death of multiple individuals causes a chain reaction due to the release of multiple toxic compounds.

3.3.3 Phosphate

The maximum dose of phosphates removed within the space of one tidal cycle was 15 ppm (Fig 3.4, day 15). We did not dose phosphates any higher than this as it was dosed in a nitrate phosphate solution and we reached capacity for nitrate removal before phosphate. Whilst our experiment did not detect the true maximum capacity within one

tidal cycle, we did detect the total capacity for phosphate of the single use beads before they needed replacing. The single use beads were replaced after they failed to remove all the phosphate in the system on day 13 (Figure 3.4). Prior to this, they had been subjected to a cumulative amount of phosphate equivalent to 55 ppm, and 5 ppm remained in the system. On day 18, we found the same result with the fresh beads that were replaced at day 13, again after a further cumulative 55 ppm had been passed through the system. Beads were not replaced after day 18, nor was any additional phosphate added, but we found no further rise in phosphate levels, thus confirming that the beads where not leaking phosphate back into the system.

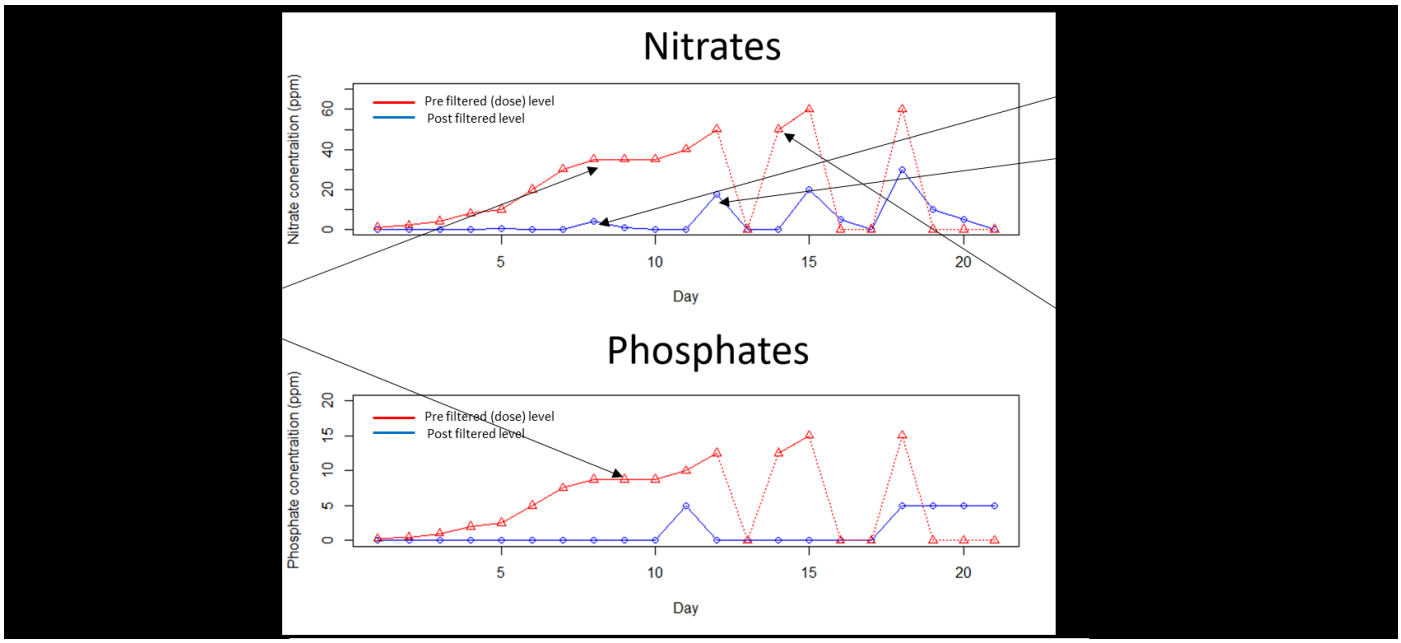

Figure 3.4 Nitrate and Phosphate levels pre and post filtered at increasing concentrations over time

3.4 Discussion

We have designed and built a system that can accurately replicate real life tidal cycles whilst simultaneously filtering high levels of nutrient additions, using a recirculating body of water. Our system accurately predicted a real life tidal cycle, within 1 cm of those provided by the BODC (BODC, 2016), an organisation who is control of the main tidal station Liverpool Gladstone dock from which the metrics to generate these tides were taken. We can also conclude that the system accurately replicated this tidal cycle as the water level in the experimental tanks for all 44 tides measured over the one-month period where within 1 cm of the expected values based on the tidal calculations.

The test of our filtration system showed that it was able to consistently filter nitrate levels of 35ppm within the space of one tidal cycle. Similarly, we were able to remove high levels of phosphate, with the filter system as described able remove 15 ppm within one tidal cycle. Levels of 50 ppm and 15 ppm N:P far outstrip those found in coastal waters showing symptoms of eutrophication. For example, (Ignatiades et al., (1992) characterised the nutrient concentrations in eutrophied water bodies. The highest nutrient concentrations from all samples were found in inshore gulf waters and were between 4.25-5.5 ppm for nitrates and 0.9-1.18 ppm for phosphates. Similarly, our filtration system was able to cope with nutrient concentrations equivalent to some of the most heavily polluted inland lakes, where nutrient concentrations are usually higher than in marine systems. Lake of Bhopal, India, has abnormally high phosphate levels at 16 ppm and Lake McIlwaine, Zimbabwe has recorded nitrate levels of 40 ppm (Marshall and Falconer, 1973). The system as described was originally designed to handle nutrients

loads of 35 ppm 15:1 of N:P in order to test the effects of nutrient addition on saltmarsh plants using similar levels of nutrient enrichments as used by previous field studies (Deegan et al., 2012). We can conclude that the system was more than adequate for our initial aims and would also be suitable for use in experiments that measured extreme nutrient levels.

A limitation of the current system was the need to replace the phosphate absorbing media after every 55 ppm in 1000 litres of water. Whilst the modular design of the filter system makes replacing the beads a simple process, any disruption to the system does present a risk of damaging the sensitive denitrifying bacterial colony that compose the biological filtration element. The filter system we used was modular so has the potential to be adapted for a range of uses. The system as described was designed to handle moderate to high levels of nitrate and phosphate inputs whilst maintaining a control tank with no detectable inputs. By changing the filtration media, the system could be altered to filter different levels or chemicals depending on the experimental requirements. For example, filters designed for aquariums are available for the removal of heavy metals, chloramines and tannins. As previously mentioned, the filtration design does not impact the flow of water responsible for tidal replication in our system. This also means that if the filtration system we have described is unsuitable for a particular experiment it can easily be replaced with a more suitable filtration method, as long as it has an inbuilt method of retuning water back to the main sump after filtration.

The closed water supply of our system represents a significant improvement over the original designs of the system described in Miller and Long, (2015). The main advantage to this is that it requires significantly less water over the course of the experiment compared to the non-recirculating system of Miller and Long, (2015). Using their original design, our most conservative estimate for the amount of water required for the test of nutrient removal as described in this study would have been 20,000 litres compared to the 1,200 litres in our updated design. This reduction in water usage has two main benefits. Firstly, it greatly reduces the effort and potential sources of error when controlling for different levels of water parameters such as pH, temperature, salinity and nutrient levels. Secondly, the system is designed to replicate a tidal cycle and so in most cases, saline water will need to be used in the system. Our experiment was conducted inland in Manchester, UK, at a facility without access to seawater on demand. At current prices, seawater is £0.30 per litre, excluding any storage or transport costs, and so the water uses of the original design would total a minimum of £6,000 compared to £300 in our experiment. This represents a significant cost saving in the space of one short 20 day experiment.

Our tidal inundation machine opens up several new possible lines of research, as well as the possibility to improve previous techniques. It is hoped that the increased practicality of such a system will allow for more experiments looking at the effect of tidal inundation to include a more realistic representation of the effect of tide. This is particularly important as sea levels are rising and its potential effects are a major point of research within the scientific community (1.2 million papers referring to the subject published in the last decade, searched on google scholar in July 2019). The machine will also allow

for experiments that look at both tidal inundation and nutrient addition in a controlled environment and allow us to disentangle their interactions. This has so far proven elusive in the natural environment, despite long-term large scale experiments such as (Johnson et al., 2016). In addition to controlling nutrient levels, being able to replicate tidal conditions in a controlled laboratory setting allows for greater control of other variables that are difficult to replicate in the natural environment, such as genetic identity. The machine provides huge flexibility in experimental design while also providing an economic solution for multiple and long term experiments.

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Chapter 4: Response of different genotypes of two saltmarsh grasses, *Puccinellia maritima* and *Festuca rubra*, to increased nutrient concentrations and sea level rise

4.0 Abstract

Saltmarshes are under increasing threat from rising sea levels and nutrient enrichment from anthropogenic sources. We need to understand how saltmarshes will respond to these stressors, and to the interaction between them, both in terms of species composition and functional traits, so we can best predict how these important habitats will change in the future. This is complicated by the fact that we do not understand the relative influence of genotype on survival and traits. In this study, we use a newly developed simulator of real-life tidal inundation and control of nutrient concentrations in a three-factor glasshouse experiment. We tested the effects of a simulated 30 cm rise in sea level and increased nutrient concentrations on the survival and functional traits of two saltmarsh grasses, *Puccinellia maritima* and *Festuca rubra.* We also tested for the effects of genetic composition by using five different clonal strains of each species within the experiment. Increased flooding reduced the survival of *Festuca rubra* but not of *Puccinellia maritima*, although overall survival of *Puccinellia* was low. Importantly, for both species clones of different identities responded differently to the combination of flooding and nutrients, highlighting the important role genetics play in intraspecific variation. Nutrient addition mitigated the stress of increased flooding due to low elevation in *Festuca rubra.* As observed as an increase in above ground biomass compared to the low elevation no nutrients treatment. There was a varied response of other traits to nutrient enrichment and flooding. Overall, these results demonstrate the complex intraspecific response of two saltmarsh species to changing environments.

4.1 Introduction

Elevation plays a major role in determining saltmarsh plant distribution (Gray, 1992). Changes in elevation across the marsh lead to changes in the duration and frequency of inundation, which in turn drive important environmental variables, such as salinity and redox potential (Davy et al., 2011). These are further modified by sediment characteristics (Crooks et al., 2002) and topography (Wang et al., 2007). Differential tolerance of saltmarsh plant species to elevation and associated environmental drivers results in the clear vegetation zones often observed in saltmarsh (Davy et al., 2011; Xie et al., 2019). The influence of flooding is more complex than a simple linear change in elevation and tidal inundation, as it has been shown that localised changes in micro topography can lead to changes in redox potential of the soil regardless of the macro-scale elevation on the marsh (Mossman et al., 2019).

Flooding regime can also affect the supply and thus concentration of nutrients within the marsh. Large amounts of inorganic nitrogen are brought in at high tide and organic forms leached out as it recedes (Childers et al., 1993). This process has been shown to be more dynamic in the low marsh than the high marsh and heavily dependent on the hydrological regime of the site (Whiting et al., 1989). In addition to elevation and topography, nutrients concentrations can singularly influence saltmarsh plant distribution. Bertness et al., (2002) showed how an influx of nutrients caused by local human activities can change the competitive balance of saltmarsh plant communities leading to a change in overall vegetation structure. This builds upon previous works that

have demonstrated the role of nutrient enrichment in changing the relative dominance of species. For example, Kiehl et al., (1997) found that experimental nutrient addition had a negative effect on the biomass of one saltmarsh species, *Puccinellia maritima*, and a positive effect on *Suaeda maritima*, eventually leading to *Suaeda* displacing *Puccinellia* on the marsh. A field manipulation study by Levine et al., (1998) has further demonstrated how nutrient availability can modify the interspecies interactions of saltmarsh plant changing their intensity and direction. It is therefore important to understand the effects of increased nutrient levels on saltmarshes as coastal eutrophication continues to be a major global issue, with some areas seeing up a 15 fold increase in nitrogen levels over the last few decades (Howarth, 2008). However, our knowledge of the concentrations of plant-available nutrients in saltmarsh sediments is poor. Different soil types (e.g. organic vs mineral dominated sediments) are likely to contain and have available different levels of nutrients, and other factors, such as local scale microbial activity, will also alter availability. A recent meta-analysis of background nutrient levels in saltmarsh sediments found variability over several orders of magnitude (Lawrence 2018).

Another major global change that will affect saltmarsh ecosystems is sea level rise (Nicholls and Cazenave, 2010). The majority of saltmarsh in the UK, and many areas globally, are constrained by sea walls. As sea levels increase, the elevation range of the saltmarsh relative to sea level decreases as the saltmarsh cannot migrate inland past the retaining sea wall, a process known as coastal squeeze (Adam, 2002). Rising sea levels will result in an increase in the frequency and duration of tidal flooding across the marsh, with direct impacts on species abundances and distribution (Morris et al., 2009), and

therefore implications for overall ecosystem functioning (Craft et al., 2009). However, the increased frequency and duration of tidal inundations will also alter soil chemistry (Craft et al., 2009) and nutrient concentrations. Increased nutrient availability is generally thought to ameliorate the effects of other stressors, such as tidal inundation and waterlogging (Alam, 1999), and modify the response of saltmarsh plants to tidal inundation (Wong et al., 2015). There is also evidence that increased nutrients reverse the competitive dynamics of saltmarsh plants across elevation ranges leading to changes in species distribution across the site (Levine et al., 1998). One possible outcome is that increasing coastal eutrophication could ameliorate the negative effects of sea level rise on saltmarsh plants, but our current understanding of these complex effects remains insufficient to allow for accurate predictions.

Genetic composition has the potential to influence plant responses to environmental conditions. Different genotypes of *Spartina alterniflora* have been shown to be structured within elevation ranges, leading to changes in growth patterns and colonisation success (Proffitt et al., 2012). This can ultimately shape overall plant communities, with a particularly strong influence on the formation of newly developed saltmarsh (Proffitt et al., 2005), and influence on ecosystem functioning, particularly in dominant species (Seliskar et al., 2002). Clonal reproduction of only the most well adapted individuals could lead to limited genetic diversity within extreme habitats, such as most saline areas of saltmarsh (Richards et al., 2004). In contrast, phenotypic plasticity in response to environmental stressors may allow individuals to persist without genetic adaptation (Richards et al., 2010). Understanding the role of genotype in driving responses to environmental conditions across a variety of saltmarsh species is vital if we

are to restore and maintain valuable saltmarsh habitats in the face of environmental change.

The aim of this study is to test the combined effect of sea level rise and increased nutrient concentrations on saltmarsh plant functional traits and the differences in response between genotypes. To do this we use a three-factor experiment investigating the relative effects of increased tidal inundation, nutrient concentration and genetic identity on the survival and growth of two common saltmarsh grass species, *Puccinellia maritima* and *Festuca rubra*. Using a newly developed tidal inundation machine with the capability to control nutrient concentrations and tidal cycles, we subjected individuals to differences in both frequency and duration of tidal inundations as would be experienced in a natural environment. This allowed for a realistic replication of the effects of tidal inundation in a controlled laboratory setting. The study had two flooding conditions, with the first replicating flooding conditions as would be experienced in low-mid marsh elevation zones and the second a predicted 30 cm increase in flooding due to sea level rise in the next 30 years (Bamber et al., 2019). We also included a nutrient limited and a nutrient enriched treatment, and used clonal individuals of separate genetic identities to investigate the relative influence of tidal inundation, nutrient enrichment and genetic identity on the survival and growth of the two grasses.

4.2 Methodology

4.2.1 Study design

Our study consisted of a three-factor experiment investigating the relative impact of elevation, nutrients and genetic identity on the survival and functional trait response of saltmarsh plants. The two study species *Puccinellia maritima* and *Festuca rubra* were grown in a glasshouse under simulated tidal conditions and exposed to either relatively high or low tidal inundation conditions and in the presence or absence of nutrients. In addition to this, all individuals in the experiment were grown from single tillers, removed from one of five parent plants, so had one of five separate genetic identities.

Puccinellia maritima and *Festuca rubra* were selected as they are found across a wide range of conditions on saltmarsh, but have an overlapping elevation range (Gray and Scott, 1977). They were also selected because they are grasses and could be easily propagated to establish clonal lineages from source individuals. All plants were sourced from RSPB Marshside saltmarsh along the Ribble estuary, UK. Five individuals from each species were taken from across the marsh from a minimum of 200 m apart, and with an attempt to gather individuals from a wide range of observed elevation and waterlogging conditions. As both species form widespread clonal mats it is impossible to identify individuals in the field, we used a two-stage propagation method to establish clonal lineages for use in the experiment. Firstly, we removed a single tiller from each of the five field-sourced plants. These individual tillers were then grown for three months in

separate pots in order to establish plants with a known single genetic source. These pots contained a 50:50 sand and loam (John Innes No2: Nutrient content N 312 gm^3; P 156 gm^3) mix and were watered periodically (minimum once every 3 days) with freshwater. Single tillers from these plants were then used in the experiment.

Each individual used in the experiment consisted of a single tiller with root stock. Individuals were planted in a 7 x 7 cm square pot filled with a 50:50 sand and loam mix. Before the start of the experiment, plants were watered daily with fresh water to allow for acclimation to the new pot and reduce the chance of any adverse effects from transplantation influencing the results of the experiment. After this acclimation period (1 week), plants were placed into the experimental tanks. For each species, 16 replicates of each of the five genetic identities were placed in the nutrient-enriched tanks and 16 replicates placed in the nutrient-limited tanks. Of these 16, eight were placed 10 cm above the base of the tank (low elevation) and eight 40 cm above the base (high elevation), selected to represent a 30 cm rise in sea level as predicted by the year 2050 (Bamber et al., 2019). This gave eight replicates for each combination of elevation (high/low) nutrient enrichment and genetic identity. In total, there were 320 replicates with eight replicates in each of the four treatments (32) and five different genetic identities (160) repeated for two different species (320). Position and tank designation were randomised to avoid any effect of experimental tank outside of nutrient enrichment status, with 80 individuals per tank. This methodology included no sediment accretion and thus assumes a static elevation of saltmarsh over the time period, and thus is an experimental test of *relative* elevation. At many saltmarshes sediment accretion will occur in addition to sea level rise, resulting in negligible relative sea level

rise. However, at sediment-limited locations, a net sea level rise of 30 cm is likely. It is also important to note that the nutrient content of the sites may be heavily influenced by both the quantity and source of any sedimentation.

Plants were grown in a tidal inundation machine (TIM) described in Chapter 3. This machine replicates a true tidal cycle in real time, filling and emptying two sets of conjoined experimental tanks in line with the tidal cycle programmed. The system also contains a filtration system capable of removing nitrates and phosphates so that the water entering the tanks on each tide is free of any nutrients. This allows for selective dosing of one set of tanks with nutrient solution and ensuring the other stays uncontaminated, whilst allowing for the use of a single body of recirculating water.

The experiment ran for three months between June and August 2018. TIM was set to reproduce a tide from Liverpool Gladstone dock (the nearest tide station with available data to where source plants where collected) for this time. Half of the four experimental tanks were dosed with 30 ppm (4:1 N: P) ammonium nitrate, ammonium phosphate dibasic solution at high tide each day and the other was left untreated. The filtration system as described in Chapter 3 ensured that the two undosed tanks were left nutrient limited. The simulated elevation range of the marsh was such that all individuals would be submerged by all but the smallest tides, but for different amounts of time depending on their elevation within the tanks.

4.2.2 Sampling

After the three-month growth period, five measures of each plant were taken; survival, height (mm), width (mm), above ground biomass (mg) and below ground biomass (mg). Any individuals that died during the first month of the experiment were removed to avoid any breakdown of organic matter influencing the nutrient enrichment status of their respected tanks. No measurements, except survival, were recorded for these individuals. For the individuals remaining in the experimental tank, we took all measures at the end of the experiment, regardless of survival status as long as there was appropriate plant material remaining to measure.

All trait measurements were taken as per methods described in Cornelissen et al., (2003). Height (mm) was measured from the base of the plant to the tallest single tiller and width (mm) was taken from the widest part of the plant. Above-ground biomass was calculated by removing all material from above the soil line and drying at 90°C for 12 hours before weighing. Below-ground biomass was measured by washing all soil material through a series of graduating sieves (smallest being 2 µm) and removing the roots with fine tweezers. Root material was then dried and processed in the same manner as for above ground biomass.

4.2.3 Data analysis

We investigated survival response for each species separately. For each species we used a series of chi-squared tests to examine differences in survival response to nutrient concentrations, elevation and the combined effects of each of the two nutrient and elevation treatments together. We also tested each genetic identity separately to uncover any genotype-specific responses to the elevation and nutrient treatments. For each genetic identity, we performed three chi-squared or Fisher's exact tests, depending on suitability, to test for differences in response to nutrients, differences in response to elevation and differences in response to the combination of nutrients and elevation together. To account for type one errors from the multiple comparisons of the same dependent variable, we applied a Bonferroni correction and used the adjusted p values to infer significance for each statistical test. Table 4.1 contains the list of adjusted p values for each test and each species.

Table 4.1 Bonferroni corrected P values for the chi squared and Fishers exact test on survival response of Festuca rubra and Puccinellia maritima. The total number of tests per species is six, but this varies between chi-squared and Fishers exact tests due to the number of individuals surviving.

	Chi squared tests			Fishers exact test		
	N Tests	Unadjusted p	Adjusted p	N Tests	Unadjusted p	Adjusted p
Festuca rubra	5	0.05	0.01	1	0.05	0.05
Puccinellia maritima	3	0.05	0.017	3	0.05	0.017

The effects of elevation, nutrient addition and genetic identity on plant height, width, and above and below ground biomass were investigated using a series of three-way ANOVAs with all interaction terms included. Despite attempts to transform the data to meet the requirements for the three way ANOVAs with interaction terms, not all data fit the requirements for normality. However, ANOVAs are known to be reasonably robust when used with non-normal data and with no equivalent non-parametric test available, we concluded that this remained the most appropriate option for statistical analysis of our data.

4.3 Results

4.3.1 Survival

Overall survival was higher in *Festuca rubra*, with 98 alive and 62 dead by the end of the experiment, compared to 33 alive and 127 dead in *Puccinellia maritima.* Elevation had a significant effect on survival of *Festuca*, with survival being reduced in the low treatment (43 dead in the low treatment and 19 in the high treatment; $\chi^2 = 13.93$, df = 1, $p < 0.001$).

There was no impact of nutrients (χ^2 =0.658 df =1, p=0.417) or genetic identity (χ^2 = 10.17, df = 4, p = 0.038 – p value above Bonferroni correction of 0.01; see also Appendix 4.1) on survival of *Festuca rubra*. There was no difference in the survival of *Puccinellia* between elevation treatments (χ^2 = 0.15, df = 1, p = 0.696), genetic identities (χ^2 = 2.17, df = 4, p = 0.710) or nutrient treatment (χ^2 = 3.818, df = 1, p = 0.05 – p value above Bonferroni correction of 0.017).

Although there was no overall effect of genetic identity on survival for either species, we did detect differences in survival response between the individual genetic identities used in the experiment. There was a significant difference in the survival of *Puccinellia* genetic ID 5 in the different nutrient treatments, with no individuals surviving without nutrients present and half the individuals surviving when they were (p = 0.002) (Figure 4.1). This response was different to the other genetic identities, where none showed a significant response (Fisher's exact test: genetic ID 1 p = 0.519; genetic ID 2 p = 0.440; genetic ID 3 p = 0.600; genetic ID 4 p = 1.00). There was also a significant difference in response of *Puccinellia* genetic ID 5 to the combination of elevation and nutrients together (p= 0.007), although this appears to be as a result of nutrient concentration as none survived under the low nutrient conditions in either elevation treatment (Figure 4.1).

For *Festuca rubra*, genetic ID 1 had increased survival in the high elevation treatment compared to the low treatment (Fig. 4.2; 13 survived in the high and four in the low treatment, χ^2= 8.031, df = 1, p = 0.005). There was also a significant difference in the

survival response of *Festuca rubra* ID 1 to the combination of nutrient and elevation treatment ($p=0.002$). In the high elevation treatment, nutrients appeared to have little impact on survival with two dying with no nutrients present and one dying when they were. In the low elevation treatment, all eight died without nutrients present but only half died when they were present.

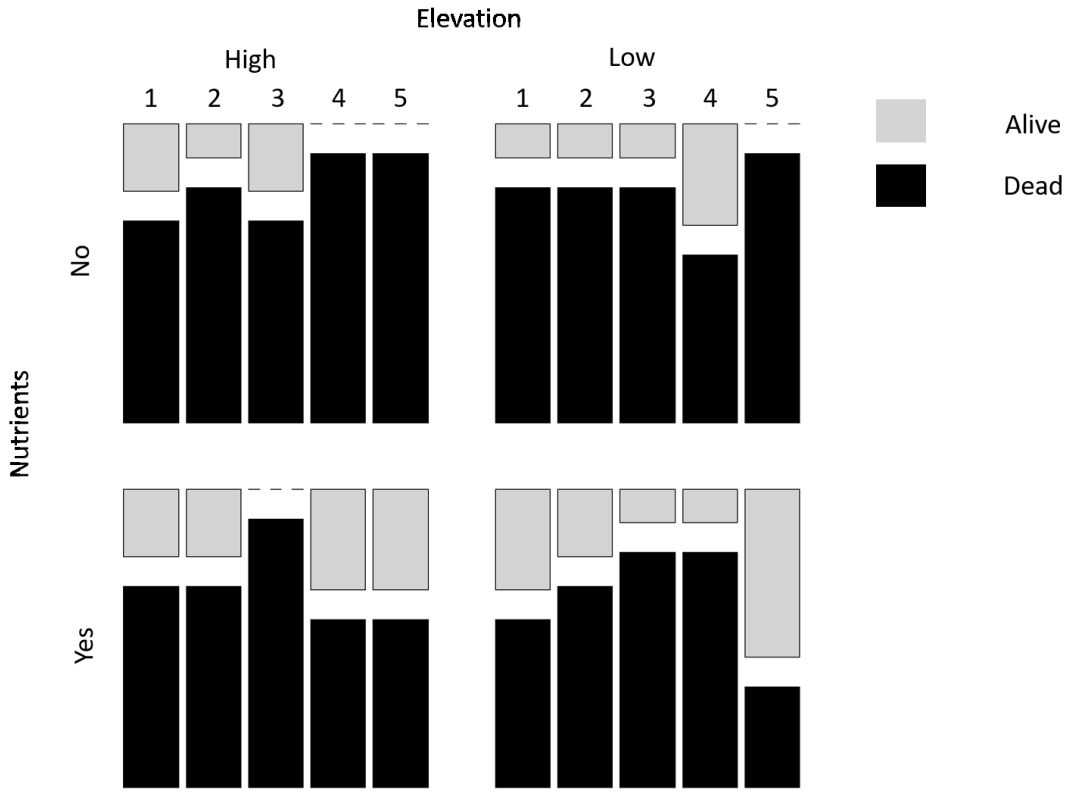

Figure 4.1 Survival of different genetic clonal strains of Puccinellia maritima (genetic ID 1-5) between different combinations of high and low elevation and in the presence or absence of nutrients

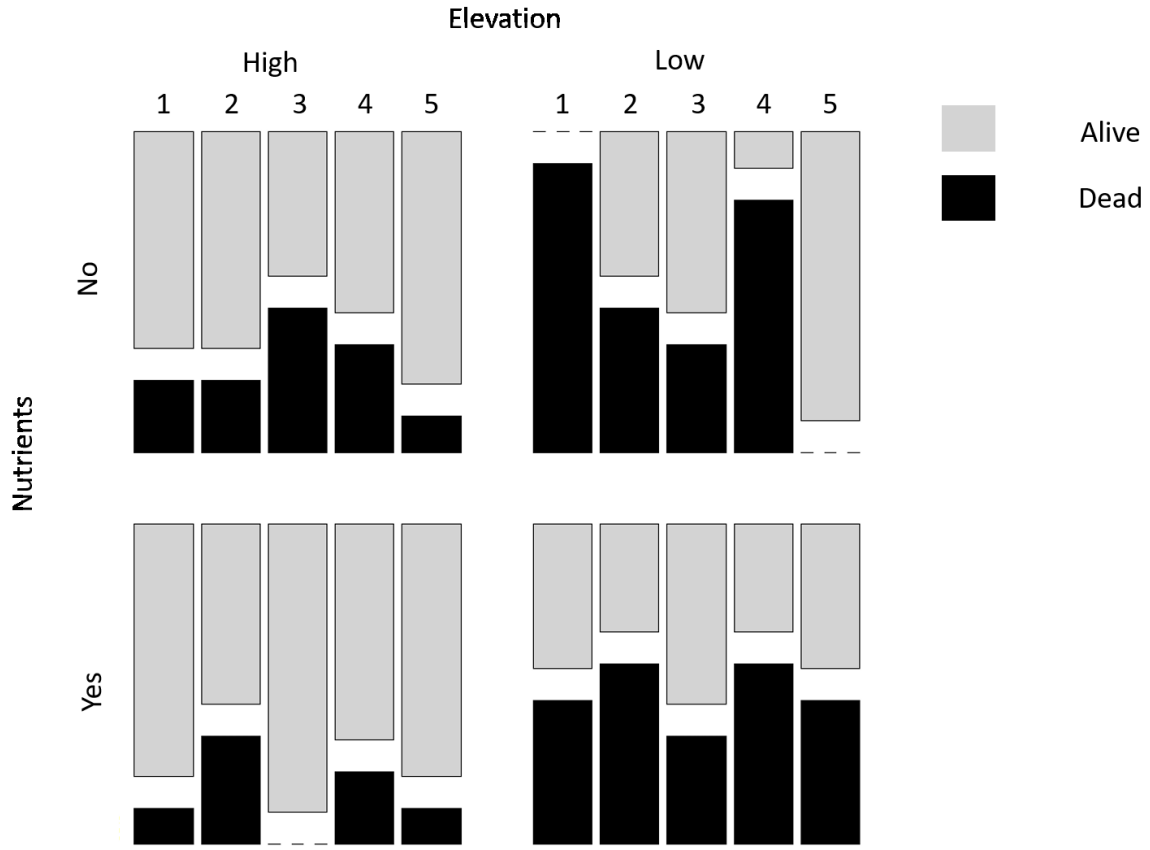

Figure 4.2 Survival of different genetic clonal strains of Festuca rubra (genetic ID 1-5) between different combinations of high and low elevation and in the presence or absence of nutrients

4.3.2 Functional traits

Puccinellia maritima was significantly taller in the high elevation treatment compared to the low elevation treatment ($F_{1,56}$ = 4.543, p = 0.039, Figure 4.3a). There was no effect of elevation, nutrients or genetic identity on *Puccinellia* width, above and below ground biomass or root mass (Figures 4.3 b-d). A full list of statistical tests and results are presented in Table 4.2.

Table 4.2 Full list of test statistics for three way ANOVAs including all interaction terms on the response of functional traits in different genetic identities of Puccinellia maritima to different elevation and nutrient treatments.

Puccinellia maritima							
Height				**Width**			
N= 57	Main Effects			N= 57	Main Effects		
	Df	F value	Pr(>F)		Df	F value	Pr(>F)
Elevation	1	4.543	0.039	Elevation	1.000	0.328	0.570
Nutrients	1	1.132	0.294	Nutrients	1.000	2.892	0.097
Genetic.ID	4	1.776	0.153	Genetic.ID	4.000	1.426	0.243
	Interaction Effects				Interaction Effects		
Elevation:Nutrients	1	1.113	0.298	Elevation:Nutrients	1	2.130	0.152
Elevation:Genetic.ID	4	1.166	0.340	Elevation:Genetic.ID	4	0.957	0.441
Nutrients:Genetic.ID	3	0.369	0.775	Nutrients:Genetic.ID	3	0.536	0.660
Elevation:Nutrients:Genetic.ID	2	2.483	0.096	Elevation:Nutrients:Genetic.ID	2	0.227	0.798
Residuals	40	NA	NA	Residuals	40	NA	NA
Dry Weight				**Root mass**			
N= 62	Main Effects			N= 61	Main Effects		
	Df	F value	Pr(>F)		Df	F value	Pr(>F)
Elevation	1	2.131	0.151	Elevation	1	1.692	0.200
Nutrients	1	0.235	0.630	Nutrients	1	0.201	0.656
Genetic.ID	4	1.558	0.202	Genetic.ID	4	0.471	0.757
	Interaction Effects				Interaction Effects		
Elevation:Nutrients	1	0.679	0.414	Elevation:Nutrients	1	0.987	0.326
Elevation:Genetic.ID	4	1.166	0.338	Elevation:Genetic.ID	4	0.565	0.689
Nutrients:Genetic.ID	3	0.903	0.447	Nutrients:Genetic.ID	3	0.400	0.754
Elevation:Nutrients:Genetic.ID	2	0.479	0.623	Elevation:Nutrients:Genetic.ID	2	0.804	0.454
Residuals	45	NA	NA	Residuals	44	NA	NA

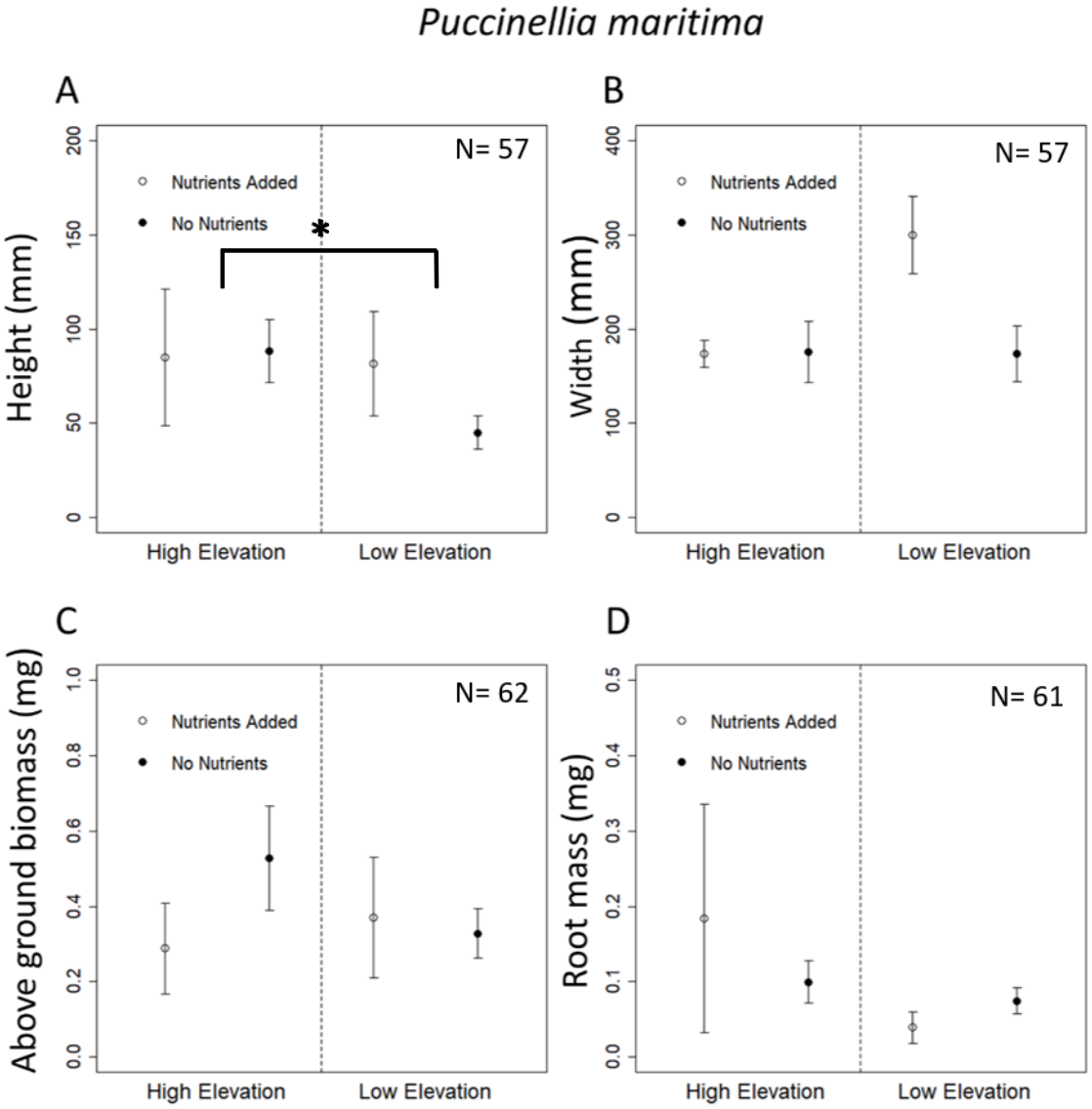

Figure 4.3 Height, width, above and below ground biomass response of Puccinellia maritima in response to differences in elevation and nutrient concentrations, including the response of distinct genetic identities. Points are the mean and error bars denote SE.

Festuca rubra showed no response in height to elevation ($F_{1,108}$ = 0.001, p = 0.972), nutrients ($F_{1,108}$ = 0.595, p = 0.595) or genetic identity ($F_{4,108}$ = 1.354, p = 0.256). However, there was a significant interaction between nutrient addition and elevation on its height ($F_{1,108}$ = 5.388, p = 0.023, as denoted by the solid lines between points in Figure 4.4a); *Festuca rubra* was taller without nutrients in the high treatment and taller with nutrients in the low treatment. These results were mirrored in the above-ground biomass with no significant response to elevation ($F_{1,116}$ = 1.958, p = 0.165), nutrient addition ($F_{1,116}$ = 1.216, p = 0273) or genetic identity ($F_{4,116}$ = 0.123, p = 0.974), but there was a significant interaction between elevation and nutrients ($F_{1,118}$ = 7.974, p = 0.006) with plants having greater above-ground biomass with nutrients in the low treatments and less with nutrients in the high treatment (Figure 4.4c). Neither elevation ($F_{1,124}$ = 3.324, p = 0.071) nor nutrients ($F_{1,124}$ = 0.372, p = 0.543) had a significant effect on below ground biomass. Below-ground biomass was significantly different between the different genetic identities ($F_{4,124}$ = 2.763, p = 0.031). Genetic ID 4 had significantly more root mass than genetic IDs 2, 3 and 5 (Figure 4.4d, Table 4.3).

Table 4.3 Full list of test statistics for three-way ANOVAs including all interaction terms on the response of functional traits in different genetic identities of Festuca rubra to different elevation and nutrient treatments

Festuca rubra

Height

N= 108	Main Effects		
	Df	F value	Pr(>F)
Elevation	1.000	0.001	0.972
Nutrients	1.000	0.285	0.595
Genetic.ID	4.000	1.354	0.256
	Interaction Effects		
Elevation:Nutrients	1.000	5.388	0.023
Elevation:Genetic.ID	4.000	0.187	0.945
Nutrients:Genetic.ID	4.000	0.342	0.849
Elevation:Nutrients:Genetic.ID	3.000	0.633	0.596
Residuals	89.000	NA	NA

Width

N= 108	Main Effects		
	Df	F value	Pr(>F)
Elevation	1.000	2.732	0.102
Nutrients	1.000	1.418	0.237
Genetic.ID	4.000	1.477	0.216
	Interaction Effects		
Elevation:Nutrients	1.000	0.839	0.362
Elevation:Genetic.ID	4.000	0.345	0.847
Nutrients:Genetic.ID	4.000	1.286	0.282
Elevation:Nutrients:Genetic.ID	3.000	0.383	0.766
Residuals	89.000	NA	NA

Dry Weight

N= 117	Main Effects		
	Df	F value	Pr(>F)
Elevation	1.000	1.958	0.165
Nutrients	1.000	1.216	0.273
Genetic.ID	4.000	0.123	0.974
	Interaction Effects		
Elevation:Nutrients	1.000	7.974	0.006
Elevation:Genetic.ID	4.000	0.982	0.421
Nutrients:Genetic.ID	4.000	1.573	0.188
Elevation:Nutrients:Genetic.ID	3.000	0.428	0.734
Residuals	98.000	NA	NA

Root mass

N= 125	Main Effects		
	Df	F value	Pr(>F)
Elevation	1.000	3.324	0.071
Nutrients	1.000	0.372	0.543
Genetic.ID	4.000	2.763	0.031
	Interaction Effects		
Elevation:Nutrients	1.000	0.210	0.648
Elevation:Genetic.ID	4.000	0.463	0.763
Nutrients:Genetic.ID	4.000	1.172	0.327
Elevation:Nutrients:Genetic.ID	3.000	0.726	0.539
Residuals	106.000	NA	NA
	Genetic ID Pair wise comparrisons		
	SE	df	P
1_2	0.0573	102	0.6221
1_3	0.0578	102	0.8129
1_4	0.0607	102	0.3983
1_5	0.0558	102	0.7377
2_3	0.0543	102	0.9974
2_4	0.0573	102	0.812
2_5	0.0521	102	0.9992
3_4	0.0578	102	0.8324
3_5	0.0521	102	1
4_5	0.0558	102	0.8181

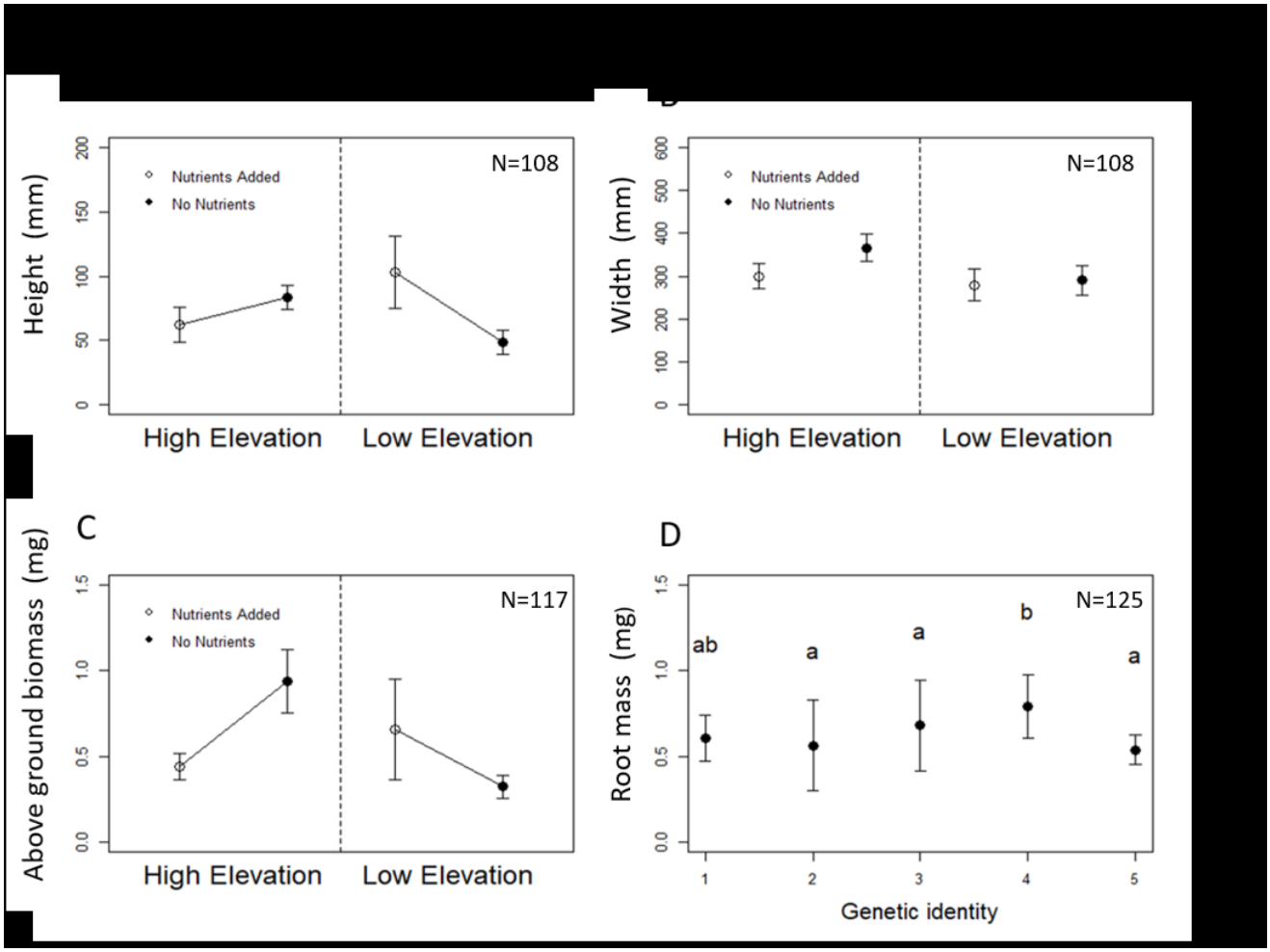

Figure 4.4 Height, width above and below ground response of Festuca rubra in response to differences in elevation and nutrient concentrations, including the response of distinct genetic identities. Points are the mean and error bars denote SE. Connected lines between points denote the presence of significant interactions between response to elevation and nutrient treatments.

4.4 Discussion

We found that the combined effects of predicted nutrient increases and sea level rise will have an impact on our two study species and that responses were genotype-specific. All treatments had at least one effect on survival, although not for all of the genetic identities or on all species used in the study. We did not detect a difference in overall survival response of Puccinellia *maritima* although we did detect it in specific genotypes. *Festuca rubra* overall survival was influenced mostly by elevation. We also found differences in survival response of the different genetic identities to the flooding and nutrient treatments for both species, suggesting that genetic identity could play a key role in determining viability of individuals under extreme environmental conditions. Of the individuals that survived long enough to produce measurable growth at the end of the experiment, we found evidence for differences in the functional traits of both species in response to the different treatments in our study.

4.4.1 Survival response

Overall, survival for both species was low, particularly in *Puccinellia maritima* with more than half the individuals dying during the experiment. Frequency of inundation, and factors influenced by it such as sediment waterlogging, can determine the distribution of both species (Gray and Scott, 1977). However, it was not expected that more *Puccinellia maritima* plants would have died compared to *Festuca rubra* as *Festuca* is usually found at higher elevations compared to *Puccinellia* and is less tolerant to waterlogged conditions (Gray and Scott, 1977). Our experiment simulated conditions on

the low marsh so we would have expected lower survival in *Festuca* than *Puccinellia*. Indeed, while more *Festuca* survived overall, we did find that *Festuca rubra* was more sensitive to changes in elevation than *Puccinellia maritima* as it survived better with less flooding (high treatment). As *Puccinellia maritima* did not respond significantly to any of the treatments in our study it is possible that there were other factors in the experimental design, such as the cloning and planting procedure that caused it to have lower survival than *Festuca rubra.* As we also showed significant differences between the different genotypes in the study, it is also possible that the genotypes selected are also influencing this result.

We found no effect of nutrients on the overall survival of *Puccinellia maritima* or *Festuca rubra.* This was a contrasting response to nutrients to that found in a field study by (Kiehl et al., 1997), who found that *Puccinellia* growth was restricted at lower elevation when nutrients where added and that it was out competed by other species. As we tested each species in isolation, our results would suggest that the effect of nutrient concentrations in isolation were not enough to influence the viability of our two study species at different elevation ranges. This is in contrast to previous research that have shown that nutrients do have a significant impact on the vegetation structure of a saltmarsh, with saltmarshes containing similar nutrient concentrations having similar species assemblages, an effect that cannot be explained by the similarity of other variables alone (Bertness et al., 2002). One of the reasons for this effect may be that nutrient levels change the interspecific interactions on the marsh, ultimately leading to expansion of some species range and displacement of others (Kiehl et al., 1997), rather than the distribution of the species *per se*. Although our study only included two species,

they are dominant species on the marsh and our results would suggest that nutrient concentrations in isolation will not significantly impact individual species survival response to increased flooding as a result of sea level rise. Instead future studies should focus on the interplay of nutrient concentrations and interspecific interactions to assess how future changes will impact marsh ecosystems.

We also found a range of responses of the different genetic identities to both elevation and nutrient concentrations. One of the extreme examples of this was 100% survival of *Festuca rubra* genetic ID 5 and 100% mortality of genetic ID 1 under low elevation-low nutrient conditions. This polarised response suggests that differences in genetic identity could potentially play a huge role in determining survival under different conditions. The idea that genetic variation of a species can be spaced along geographic and environmental gradients has been well studied in a number of systems and species, although there has been limited research in saltmarsh. For example, the widespread broadleaf tree species, *Populus trichocarpa*, has been observed to vary across its North American range with genetic differences linked to variations in light regimes (McKown et al., 2014). This phenomenon has also been found in aquatic systems with the genetic structure of anemonefish, *Amphiprion bicinctus* populations being found to vary along geographic and environmental gradients across the Red Sea (Nanninga et al., 2014). Previous research on one of our study species, *Puccinellia maritima*, has already shown that genetic populations are structured along elevation gradients within sites (Rouger and Jump, 2015). Our research shows that survival response to a combination of nutrients and elevation differed between genotypes, possibly explaining one of the mechanisms behind this structuring. Previous research has also shown that genetic

populations of *Puccinellia maritima* differ geographically across the UK (Rouger and Jump, 2014). Our results highlight the need to consider the genetic composition both within and between sites when trying to predict the possible effects of rising sea levels and nutrient concentrations suggesting that it could play a key role, necessitating the need to consider response of saltmarsh at the regional and site-specific level. As conditions change due to coastal eutrophication and sea level rise, survival of the species in an area will depend on the suitability of genotypes within the population available to colonise, potentially leading to a loss of the species entirely if there are no suitable individuals within the genetic pool. We have no way of predicting the possible consequences for saltmarsh ecosystems as a whole as we currently lack detailed genetic information for most of the plant species that inhabitant these areas.

4.4.2 Trait Response

Another possible impact of coastal eutrophication and sea level rise on saltmarsh could be changes to the valuable ecosystem functions they provide. Environmental change may influence genetic composition and could have much wider implications for changes in ecosystem processes globally as a result of climate change (Whitham et al., 2006). One of the mechanisms behind this is that plasticity in phenotypic response is a key component for survival when colonising new environments (Yeh and Price, 2004) and plasticity in response can be limited by genetic composition (Via, 1987). Under changing conditions, such as the effect of climate change, plasticity as a genetic trait is likely to be under increased selection pressure (Nicotra et al., 2010). In our study, we measured the

phenotypic plasticity as functional trait response in both our study species to investigate how they will respond to changing nutrient and sea levels as well as how these responses differed between genotypes.

For *Festuca rubra*, we found that responses of height and above ground biomass were context-dependent, with individuals being larger with nutrients present at low elevation but smaller with nutrients present at high elevation. Height and total amount of vegetation have been linked to wave attenuation, a crucial ecosystem function of saltmarsh (Möller, 2006; Anderson and Smith, 2014). This result shows that changing conditions could cause potentially complex and highly situational-specific responses of saltmarsh vegetation and thus its provision of this crucial ecosystem function. *Puccinellia maritima* only showed a response in height to elevation, being taller in the high treatment. It could be interpreted that the other treatments had no effect, but with so few surviving (n=33), it is probable that our study did not have sufficient replication to discern any differences in the other treatments and traits.

The increased above-ground biomass in response to nutrients, particularly in the low treatment, supports the effects observed across a whole saltmarsh, e.g. by Deegan et al., (2012). In the same study, they also observed a loss in below ground biomass with higher nutrients, then linked this decrease to the loss of structural integrity of saltmarsh. In our study, we did not observe any response of root mass to either elevation or nutrients, instead finding that root mass of *Festuca rubra* was influenced more by the genetic identity of the individuals. Understanding the complexities of root mass

production is extremely important as it is directly linked to soil stability (Gyssels et al., 2005) and changes can lead to loss of saltmarsh altogether (Deegan et al., 2012). Our results show that in addition to considering the effects of nutrient levels, tidal inundation and species composition it is also necessary to consider the relative response of different genotypes when predicting response to future changes in environmental conditions.

Overall, we have found that response of *Puccinellia maritima* and *Festuca rubra* to changes in elevation and nutrient conditions will be highly species and situational specific. This was true both for their survival and functional trait expression. We found no convincing evidence that increased nutrient concentrations will consistently offset the impact of rising sea levels. We also found that genetic compositions of the two species will also dictate their response to changing environmental conditions. Further to this, our results highlight the increasing need for more comprehensive research into the genetic landscape of saltmarsh. Our methodology provides a solid framework from which to study the effects of genetic identity on the response of saltmarsh plants to changing environmental conditions but we also require more studies such as Rouger and Jump, (2014) and Rouger and Jump, (2015) focusing on a range of saltmarsh species to understand how the results of further laboratory work will translate in real world ecosystems.

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Chapter 5: Differences in genetic structure of *Puccinellia maritima* between natural saltmarsh and restored saltmarsh of different ages

5.0 Abstract

It has previously been proven that restored saltmarshes do not have equivalent environmental or biological characteristics compared to natural saltmarshes. Genetics remains a severely understudied area of wetland research and we still have very little information on the genetic composition of restored compared to natural saltmarsh, nor do we understand how genetic composition develops over time in these areas. Wallasea Island is a site in the UK that has been restored in different stages, leading to a unique opportunity to study different aged restoration sites and how genetic composition develops over time. We sampled a previously studied saltmarsh grass species *Puccinellia maritima* in four different aged restoration sites (2,15,35,50 years old) as well as a historic (200 year old) natural site, all located within Wallasea Island. Genetic composition was significantly different between all of the restored sites compared to the natural site. Despite differences in composition we found that genetic diversity was not consistently different between sites of different ages or restoration status. Some genetic subpopulations that we identified where structured along elevation gradients, but others were found at the same elevations suggesting that elevation as well as other unknown environmental variables may define genetic distribution. The equivalency in genetic diversity between the youngest restoration sites and the natural site is a good indication that newly restored sites have the same levels of resilience inferred by genetic diversity.

5.1 Introduction

Restoration of saltmarsh in the UK is a vital component in the effort to stop the loss of natural habitat available in the UK. The EU Habitats Directive now requires that any area of saltmarsh lost must be replaced with one that is biologically equivalent to that which is lost (Commission, 2000). However, current restoration efforts fail to produce sites with equivalent species assemblages and we are therefore failing to meet the standards for biological equivalency as laid out in the European Habitats Directive (Mossman, Davy, et al., 2012). While the species diversity and composition of plant communities on restored and natural saltmarshes is relatively well studied (Wolters et al., 2005, Zedler et al., 2001, Bakker et al., 2002, Chang et al., 2016), other important components of diversity, such as the genetic variability within populations, are less well known. This is despite the importance of genetic composition being well understood (Hughes et al., 2008). Across all systems, genetic diversity is known to infer resilience by ensuring there is enough genotypes that can survive environmental changes (Reed and Frankham, 2003). Genetic diversity also allows individuals to colonise different environments by increasing the level of phenotypic plasticity within the population. For example, the growth and colonisation of one saltmarsh species *Spartina alterniflora* has been shown to differ across environmental gradients, as different genotypes exhibited different growth strategies allowing them to be more competitive under different environmental conditions (Proffitt et al., 2012).

Saltmarsh is under threat from a variety of influences such as rising sea levels and other changes in hydrological regimes brought about by climate change (Adam, 2002). In the

context of wider environmental change, it's important to consider the role of genetic diversity in restoration success (Rice and Emery, 2003). This includes the many facets that influence the genetic landscape such as traits, gene flow and demography in order to restore sites that have the potential to adapt to environmental change. Any changes in genetic makeup also have the potential to influence the ecological functioning of a marsh. Different genotypes have the potential to exhibit different morphological / functional traits which could ultimately contribute to changes in functioning. For example in a salt marsh setting, a genotype that exhibits smaller roots, may lead to less soil stability and more erosion, or shorter plants may contribute less to wave attenuation (Hughes, 2014).

Due to the importance of genetic diversity in ecosystem functioning and resilience, it is vital to establish if restored sites contain similar levels of genetic diversity, and similar genotypic compositions, to natural sites. Natural marshes have a much greater variation in environmental conditions than restored sites, and so contain a larger range of niches for plants to colonise (Lawrence et al., 2018). This homogeneity can reduce species diversity (Lawrence et al. in prep), but might also limit the number of genotypes if they are related to the environment. Rouger and Jump (2015) found some (albeit weak) evidence that environmental conditions (primarily elevation) could influence genetic composition of one saltmarsh species, *Puccinellia maritima*. Questions still remain as to whether restored marsh differ genetically to their natural counterparts and to what the overriding influences of genetic composition may be in these systems. As described in a meta-analysis by Mijangos et al., (2015) genetics is still severely understudied in the

context of restoration despite an increasing agreement of its importance by the scientific community.

The restoration of new saltmarsh through the breaching of a sea wall and the reintroduction of tidal flows into the area causes a very drastic change in environmental conditions. As such, a newly restored marsh is an extremely dynamic environment, but one that has conditions suitable for colonisation by saltmarsh plants immediately (Mossman; Brown, et al., 2012). These newly restored sites have little to no vegetation cover and halophytes quickly colonise (Davy et al., 2011), and such rapid colonisation may mean they are susceptible to founder effects. These founder effects infer an advantage to early colonisers, potentially resulting in longer term reductions in genetic diversity. In contrast, natural marshes establish relatively slowly through the accretion of sediment and plant species colonise when the environmental conditions become suitable to them (Chapman, 1939). Longer term filter effects of the changing environmental conditions (Grime, 1998) are therefore likely to allow for greater genetic diversity on natural marshes. This is coupled with a longer timeframe for colonisation events to occur, as many natural marshes are hundreds of years old.

Studying the effects of genetic diversity over time would be preferable but as changes to genetic diversity occur over decadal time periods this is not often feasible. The UK has restored saltmarsh that vary in age from 1 – 130 years old (Mossman, Davy, et al., 2012). These offer an opportunity to study genetic diversity over a long period through space for time substitution. Wallasea Island was restored in stages and its unique age

structure gives us the opportunity to answer some key fundamental questions on how genetic population differ between restored and natural saltmarsh and how they develop over time. This study uses the common saltmarsh grass *Puccinellia maritima* as the study organism due to its use in previous genetic work to answer some key genetic question in a restoration context.

This study aims to identify differences in the genetic population structure of restored and natural saltmarsh, and, using a space-for-time substitution, assess changes in genetic population structure over time since restoration. To achieve this, we test for differences in genetic composition and diversity of *Puccinellia maritima* in natural and restored saltmarsh of different ages. We also test for relationships between metrics of genetic composition and environmental characteristics.

5.2 Methodology

5.2.1 Sampling method

Samples were collected from saltmarsh located in Wallasea Island, Essex, UK (51.616031, 0.83481774). Wallasea Island is a large area of restored saltmarsh in Essex UK that when completed will have converted nearly 1500ha of arable land back to historic wet land, making it the largest man made nature reserve in Europe (Cross, 2017). The scale of such as project has necessitated that it be completed in stages, resulting in several patches of adjacent restored saltmarsh of different ages. In addition, there are some areas that were breached during storm events 50 years ago. There is also an area

of historic natural saltmarsh contained within the site that is at least 200 years old. This offers the unique opportunity to study how these areas develop over time without the need for long term studies as we have areas of different ages that are comparable as they are within the same geographic area and thus subject to the same climatic conditions. They are particularly suited to the study of genetic populations as they are all in very close proximity so will most likely have very similar availability of seed stock and connectivity to the adjacent natural marsh as well as each other.

We sampled five sites within Wallasea Island of different ages; 2 year old, 15 year old, 35 year old, 50 year old and a 200 year old natural saltmarsh (Figure 5.1). From each site, we took genetic samples from 50 individual *Puccinellia maritima* plants, giving us 250 individuals across all locations. Samples were taken by removing approximately five grams of live, above-ground plant material and submerging it in fine granulated silica gel within an air tight sealed bag. This was done to dry and preserve the material before processing. We used a nested design wherein we sampled a higher proportion of plants spaced close together and increasingly fewer plants as we increased the distance. We sampled an initial 20 individuals spaced 2 metres apart (or the nearest individual to the 2 metre point) within a 10m x 10m grid. We then took a further 30 samples radiating out from either side of this grid spaced 10 metres apart (or the nearest individual to 10m) giving a total of 50 samples per sample site. GPS locations were taken for each individual except for those in the 50 year old restored site, this site was sampled separately as part of an initial pilot study and no GPS measurement were recorded.

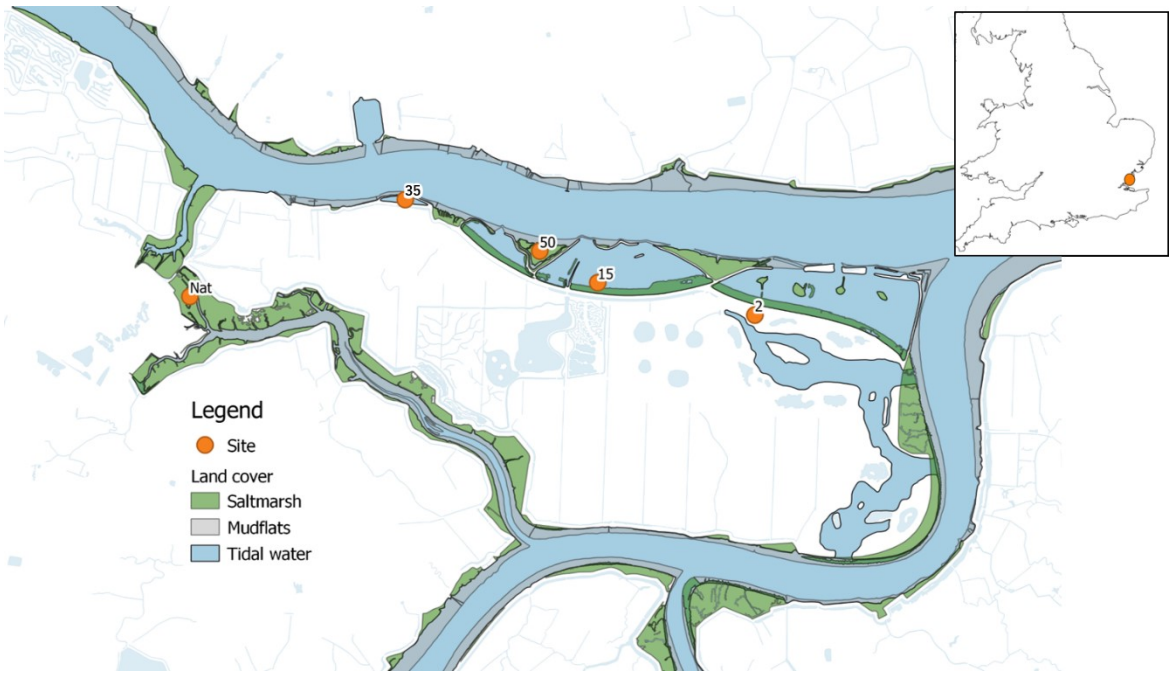

Figure 5.1 Location of Wallasea Island in the UK and the location of the five different aged sample sites within Wallasea Island

5.2.2 Sample processing

DNA was extracted using the ISOLATE II Plant DNA Kit (Bio line). Samples were then amplified using 11 microsatellites following the same protocol as described by Rouger et al., (2014). Originally Rouger et al., (2014) described the development of 12 microsatellite locations but as per Rouger and Jump, (2015) we found locus pm27 had a high amplification failure rate so we did not include this in our study. Sequencing was conducted using a capillary sequencer by University of Manchester. Scoring of alleles was done using an adapted version of the "fragman" package in R statistics software (Covarrubias-Pazaran et al., 2016; R Studio Team, 2019). UK populations of *Puccinellia maritima* are octoploidy (Scott and Gray, 1976) and the adaptations to the "fragman" package were made solely to allow for easier scoring of polyploidy individuals. Binning of alleles was done using the R package "msatalle".

5.2.3 Data analysis

5.2.3.1 Data preparation

The octoploidy nature of *Puccinellia maritima* makes scoring alleles challenging due to difficulties in distinguishing multiple alleles and in inferring allelic dosage. This also increases the likelihood of the resulting data violating the assumptions of many traditional genetic analysis methods, such as Hardy Weinberg equilibrium (Dufresne et al., 2014). In order to account for this we used the same approach as previously used in genetic studies of *Puccinellia maritima* (Rouger and Jump, 2014, 2015) and recorded alleles as either present or absent, and used methods that do not require the

assumptions of the Hardy Weinberg equilibrium. After converting alleles to binary format, the resulting matrix consisted of a list of all markers and a score of presence or absence for every allele found across the whole population for each individual.

5.2.3.2 Genetic Diversity

Allelic richness was calculated by inputting the data into the R package "adegenet" (Jombart, 2008) as a "genind" object and using the default method for allelic richness calculation parsed from the package "popgenreport" (Adamack and Gruber, 2014). In order to compare genetic diversity between sites, we first calculated Shannon diversity using each individual plant as a sample and each allele location as a separate 'species'. We then grouped individuals by site and used a Kruskall Wallis test followed by a post hoc Dunns test to compare diversity between sites. Whilst not originally designed for the purpose Shannon diversity has been used to assess diversity of alleles in challenging multiploidy species where you cannot conform to assumptions such as the Hardy Weinberg equilibrium (Fontaine et al., 2004; Babaei et al., 2012; Boggess et al., 2014).

5.2.3.3 Population analysis

To compare genetic populations between sites we first used a principal coordinate analysis (PCOA) using the binary allele data. A dice dissimilarity matrix was calculated using the same genind object as inputted into R previously, using the "dist" function of the R package "aede4" (Dray and Dufour, 2015) . The PCOA on the resultant matrix

retained 55 principal components, which accounted for >90% of the variance explained by the model.

MVabund is frequently used to model species composition because it can simultaneously model the occurrence of multiple species in the same model. We used this multivariate regression to model differences in microsatellite occurrence between individuals in the restored sites of different ages compared to the natural site (Wang et al., 2012). We also conducted a k-means clustering analysis using the package "STRUCTURE" for genetic analysis (Hubisz et al., 2009). We first used the "find.clusters" function, in the R package "adegenet", which uses a less computationally intensive method of k-means clustering analysis based on discriminant analysis of principal components (Jombart et al., 2010). This allowed us to assign an initial k cluster range of 5-11 clusters to use in STRUCTURE. Within STRUCTURE we ran a k means clustering analysis with 50,000 iterations and 10 repeats for each value of k between 5-11. The output from structure was parsed to the web utility STRUCTURE harvester (Earl and vonHoldt, 2012) which uses the Evanno method to detect the optimum number of cluster present in the samples (Evanno et al., 2005). After detection of the optimum number of clusters (7) all 10 iterations of the STRUCTURE run for this k value where parsed to the program CLUMPP (Jakobsson and Rosenberg, 2007) and then the program Distruct (Rosenberg, 2004) in order to produce a bar plot for graphical representation of the clusters within each sampling location. This bar plot shows the results of all ten iterations of STRUCTURE run at a k value of 7 using the default "greedy" option 2 and "m" 2 with 10,000 repeats of random input order. We also tested for differences in elevation, derived from LiDAR, between clusters, using a kruskal-wallis test and pairwise Wilcoxon tests. Finally we extracted the elevation for each of the genetic samples from

LIDAR imagery using the RASTER package (Hijmans et al., 2014). We created an environmental distance matrix of the difference in elevation between sampling points and geographic distance matrix with the difference between individuals. We then used mantel tests to compare correlations of genetic composition with geographic distance and elevation. Samples taken from the 50 year old site were not included in this analysis as we did not collect GPS data for this site.

5.3 Results

5.3.1 Sample size and Allele richness

Of the 250 samples collected, 30 failed to amplify successfully during sample processing leaving 220 samples for analysis (see Table 5.1 for samples sizes). Sum richness and mean richness were greatest in 50-year-old sites (415.98 and 2.05 respectively) and lowest in two year old sites (378.96 and 1.87). Although highest and lowest scores were concurrent with site age, no consistent pattern was found with the age structure of sites.

Table 5.1 Sample size, sum allele richness and mean allele richness as calculated by the R package "aedgenet" using the default ratification method

	2 year site	15 year site	30 year site	50 year site	Natural site
Sample size	49	41	44	48	38
Sum Richness	378.96	406.59	381.69	415.98	385.00
Mean Richness	1.87	2.00	1.88	2.05	1.90

5.3.2 Shannon diversity

Shannon diversity was calculated from the present or absence of microsatellite (allelic) locations for each individual. There was a significant difference in genetic diversity (Shannon diversity) between the different aged sites (Kurskall-Wallis: χ^2=15.199, df=4, p=0.004, Fig 5.2). Pairwise comparison tests showed that the 15 year old site (d=2.75, p= 0.003), 50 year old site (d=-3.16) and the natural site (d=-2.22, p=0.001) were significantly more diverse than 35 year old site. The youngest site was only significantly less diverse than the 50 year old site (d=-2.17, p= 0.189). There was no significant difference between the 50 year (d—0.749 p=0.227) or the natural (d=0.399, p-0.344) and the youngest site. There was also no consistent difference between sites based on restoration status, with the unrestored natural site not being significantly different from the 2 year old (d=0.39, p= 0.345), 15 year old (d=-0.88 ,p=0.180) or 50 year old (d=1.148, p=0.125) restored sites.

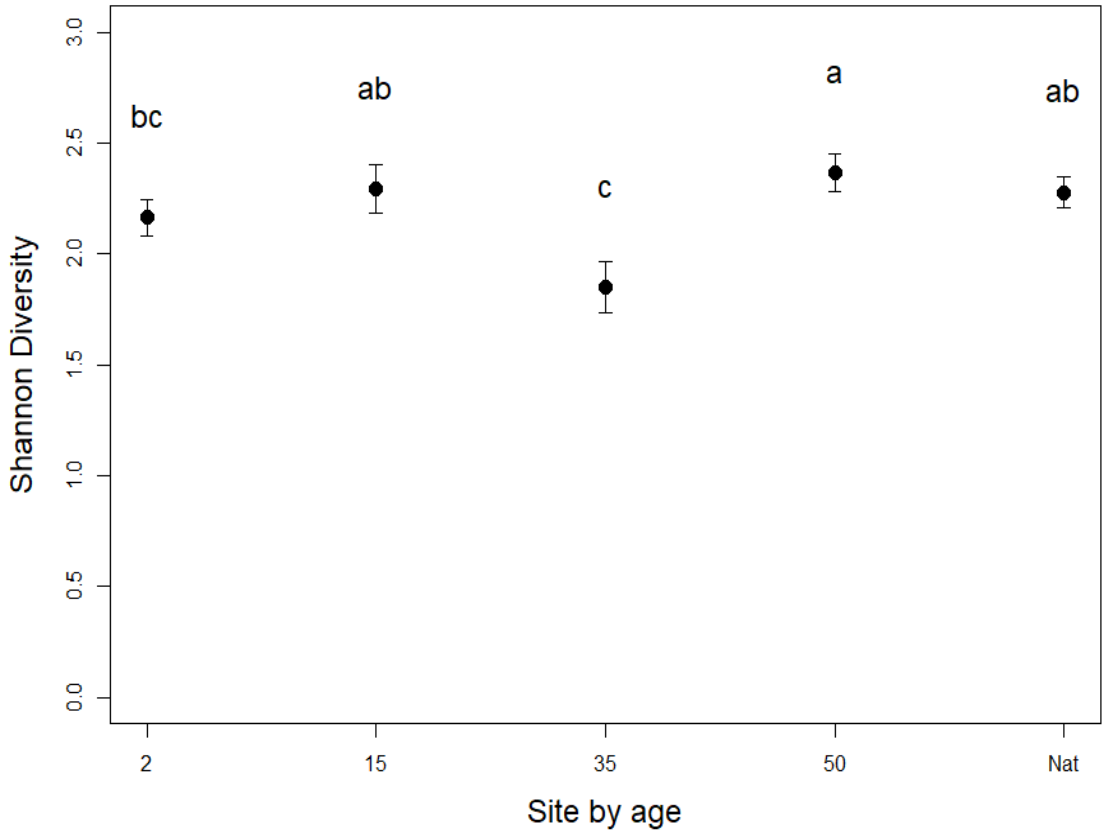

Figure 5.2 Shannon diversity between sites of different ages and restoration status located in Wallasea island UK. Nat is a 200+ year old natural saltmarsh ages 2-50 are all restored sites. Letters above the points represent significant differences. Points sharing a letter are not significantly different whilst those that do not share a letter are significantly different.

5.3.3 PCOA & Multivariate GLM

The PCOA did not produce complete separation of all the sites and showed a significant overlap between sites (Fig 5.3). Of the separation that was apparent, there did not appear to be a clear pattern relating to the age of the site. The Natural site (purple in figure 5.3) was separated from the 15 and 35-year-old sites but not the 2 or 50 year old site along axis one of the PCOA. The 15 and 35-year-old sites seemed very closely related to each other whilst the 2 year old and 50 year old site overlapped relatively evenly with all of the other sites. The multivariate GLM revealed that there was a significant difference in genetic composition between sites (dev=2744, df_1=4 df_2=219, p=0.001). Pairwise comparisons comparing the natural site to each of the restored sites revealed that all of the restored sample sites had a significantly different genetic composition to that of the natural site ([2 year old LR=574.8, p=0.003], [15 year old LR=1009.2 p=<0.001], [35 year old LR=769.0, p=0.007], [50 year old LR=325.6 p=0.003]).

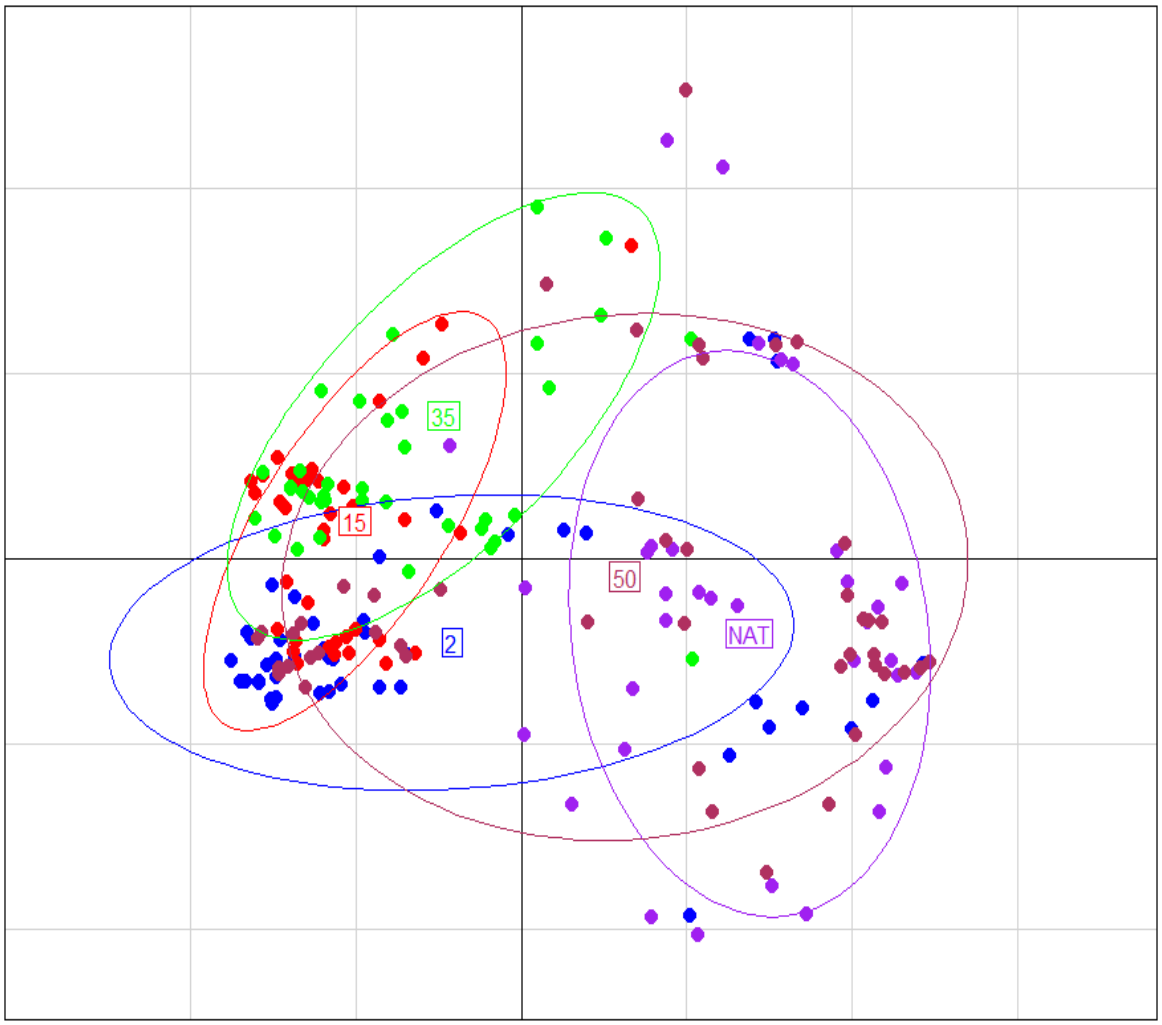

Figure 5.3 PCOA showing genetic composition of Puccinellia maritima within sites of different ages and restoration stages. Numbers at the centre of ellipsoids represent age of restored sites, NAT represents the 200+ year old natural site. Points represent individuals sampled and ellipsoids are drawn at the default 1.5 value of the positive coefficient for inertia ellipse size as calculated in the R package aed4. Total inertia of PCOA =0.42, cumulative inertia of the two plotted axis =25.69% (axis1=17.38%, axis2 =8.31%)

5.3.4 STRUCTURE Analysis

Seven clusters explained the most amount of variance within our populations (Fig 5.4). Indicating that each of the five sampled sites was not a discreet population. As expected, due to the number of clusters, Figure 5.4 shows that *Puccinellia maritima* populations are not separated between sites, instead there is a lot of mixing between the different sample sites. Qualitative assessment of the relative contribution of each cluster in Figure 4 indicates that the 50 year old and 200 year old site are very similar, as are the 15 and 35-year-old site, and that the 2-year-old site appears to be the most mixed, sharing a high proportion of clusters with the other four sites.

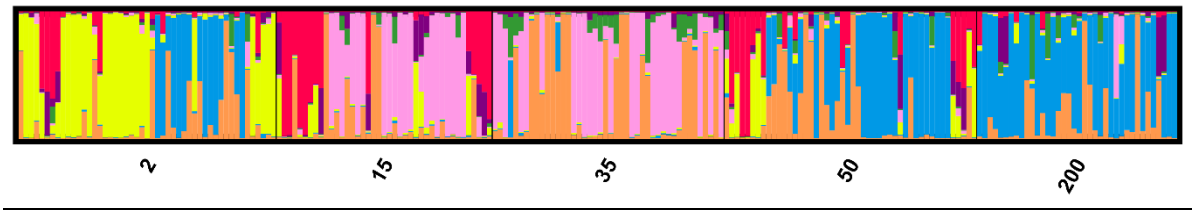

Figure 5.4 Output from CLUMP showing the average results from 10 iterations of K means clustering analysis on the allelic frequency of 220 individuals of Puccinellia maritima sourced from four restored sites of different ages (2,15,35 &50 years old) as well as a 200 year old natural site all located adjacent to each other in Wallasea Island UK

5.3.5 Effects of elevation

The results of the Kruskal-Wallis chi-squared test on the cluster assignment of each individual compared to the elevation show that some of the genetic groupings were found at significantly different elevations as seen in Figure 5.5 (Kruskal-Wallis chi^2= 39.872, df = 6, p= <0.001). A subsequent pair wised Wilcoxon test revealed that cluster

four was found in a significantly higher elevation range than clusters one (p=0.032), two (p=0.001), three (p=<0.001) and five p= (0.021). Cluster one was also found at a significantly higher elevation range than cluster three (p=0.001) The Mantel tests comparing the correlation between genetic composition and geographic distance as well as environmental distance (elevation) showed that Genetic dissimilarity was weakly correlated with environmental distance (r = 0.101, P = 0.001), but this correlation was weaker when accounting for the geographic distance between individuals (r = 0.036, p = 0.027). Genetic dissimilarity was correlated with geographic distance (r = 0.217, P = 0.001), even after accounting for environmental distance (Partial Mantel test, r = 0.196, P = 0.001).

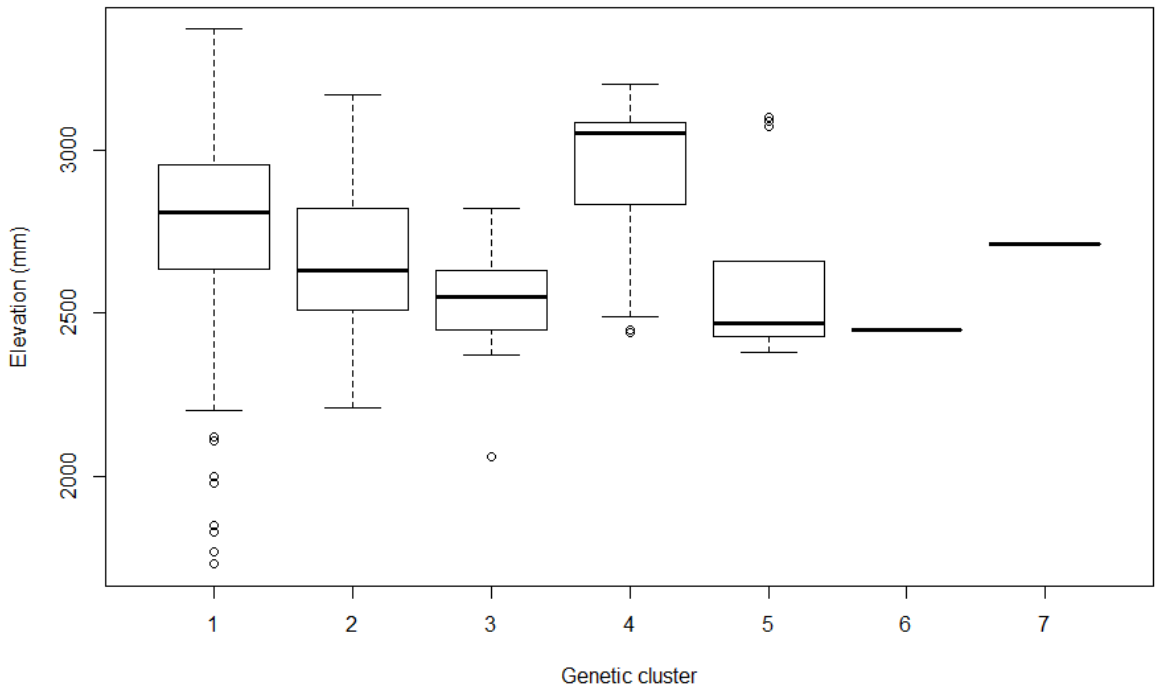

Figure 5.5 Cluster assignments for each individual from the STRUCTURE analysis plotted against the elevation that each of the samples was taken from.

5.4 Discussion

There were some significant differences in both genetic composition and genetic diversity between our study sites. Whilst all the restored sites had a significantly different genetic composition to that of the natural site, we found no evidence that the age of the restoration site influenced these differences. There was no consistent increase or decrease in genetic diversity between sites of different ages, nor was there a consistent difference in genetic diversity between the restored and natural sites. The differences in genetic diversity we did find appeared to be site-specific as there was no consistent pattern with age or restoration status. Our results suggest that the sampled populations of *Puccinellia maritima* in restoration sites have similar levels of diversity, and therefore resilience, to natural sites.

5.4.1 Genetic Diversity

Allele richness as a measure of genetic diversity is very sensitive to the presence or absence of rare alleles and can be useful to compare in populations of species were rare alleles are known to have a particular importance (El Mousadik and Petit, 1996). When the relative influence of rare alleles on the genetic structure of a species is not known, such as in our study species, there is some debate on how best to interpret results when comparing populations, but it is regarded as a good method of comparing the influence of rare alleles between populations (Rodrigáñez et al., 2008). Within our study Allele richness was fairly uniform across the study sites suggesting that the distribution of rare alleles was very similar between sites. A study by (Greenbaum et al., 2014) modelled how allele richness can reduce as a result of founder effects and then increase over time

as other genotypes migrate from seed populations. However, we found little evidence of this as the youngest site (2 years old), that would have been the most likely to exhibit founder effect, showed similar allele richness to that of a natural site that was greater than 200 years old.

Unlike with allele richness, we were able to detect differences in genetic diversity of *Puccinellia maritima* between the different sample sites using the Shannon diversity index. These differences did not appear to be structured along age gradients between the sites. This would suggest that levels of genetic diversity can establish within a very short time frame with the 2 year old site not being any less diverse than the 50 year old or the 200+ year old natural site. It would also suggest that restoration in of itself was not indicative of lower genetic diversity, as three of the four restoration sites were not significantly different to the natural population. Rouger and Jump,(2015) also compared the genetic populations of *Puccinellia maritima* between restored and natural sites, they did not account for age of the sites, and found no differences attributable purely to restoration status. Similar studies on another saltmarsh grass, S*partina alterniflora*, have also found no difference in genetic diversity between sites of different ages or restoration status (Travis et al., 2002). From the limited evidence base available we can infer that genetic diversity of saltmarsh plants can establish quickly and is also resilient to external pressures, as populations of another species *Phragmites australis* have been shown to maintain genetic diversity despite large losses in abundance and range due to a multitude of threats (Saltonstall, 2011).

Genetic diversity is thought to infer resilience in populations and has been the subject of intense research in a number of systems as we look to predict how the effects of climate change will impact the planet in the future (Hughes et al., 2003; Hughes and Stachowicz, 2004; Schaberg et al., 2008; Lin, 2011). Wetlands provide a disproportionate amount of ecosystem services for their size (Zedler and Kercher, 2005) and if this genetic diversity does indeed translate to the resilience in the face of environmental change, saltmarsh would be even more valuable in the future.

Of the differences, we did find between sites in our study they were not consistent amongst age and restoration status. This means that there was likely some other factor influencing genetic diversity within the sites. Rouger and Jump, (2015) already identified elevation as being a driver of differences in genetic composition of *Puccinellia maritima* although they did not find a significant effect of elevation in restored sites. They also found that Genetic composition was correlated with geographic distance. Our results agree with their research showing that genetic composition was correlated with geographic distance. Whilst they did not detect an effect of elevation in restored sites we did find significant correlation between elevation and genetic composition across all of our study sites, including the restored sites. This correlation was very weak (r=0.101) which may explain why it was not apparent in the previous study by (Rouger and Jump, 2015). A comparison of the response of the different genetic populations in our study, as identified by k means clustering, showed that some of the cluster assignments were differentiated along different elevation ranges whereas others were found at the same elevation range. This would suggest that only a subset of the population is sensitive to changes in elevation. This agrees with the results of chapter four of this thesis which

showed how different genotypes of *Puccinellia maritima* where much more sensitive to the effects of changes in elevation than others. It also suggests that there are other environmental factors effecting the genetic structure of *Puccinellia maritima* apart from elevation and restoration status/age and further study is needed to identify what these may be.

5.4.2 Genetic Composition

Both the PCOA and the STRUCTURE analysis revealed some separation of the study sites with some considerable overlap of the genetic composition between them. This was also confirmed by the k means clustering analysis that revealed seven different clusters best explained the composition of the five sites, whereas five distinct populations would have been best explained by five clusters. This is to be expected considering the very close proximity of the sites to each other and the potential for seeding and mixing between the populations. *Puccinellia maritima* has a UK-wide distribution and sites much further apart than in our study design have been shown to have considerable mixing between populations (Rouger and Jump, 2014). Within our study we were not able to distinguish any consistent separation or mixing based on restoration status or age since restoration. With the youngest site being the most mixed, this analysis would suggest that there is very little resistance to colonisation of any genotype to the newly restored marsh.

Despite the overlap of genetic composition between sites, the multivariate GLM test did reveal that Genetic composition of all four restored sites was significantly different to that of natural marsh. Mossman et al., (2012) showed that restored saltmarsh lacked

the same species composition of natural marsh and this contravenes the European Habitat Directive, as sites are not biologically equivalent to those they aim to replicate (Commission, 2000). Similarly, this result shows that restored marsh are also not genetically equivalent to their natural counterparts. This supports previous research as both we and Rouger and Jump, (2015) found that some populations where separated by elevation, and research by Lawrence et al., (2018) has shown that restored marshes are typically less topographically diverse than natural satlmarsh.

5.4.3 Conclusions

Genetic composition of Puccinellia maritima is not the same between restored and natural sites. Although from an ecological perspective these differences may not translate into a loss of overall fitness of the population as overall the population's maintained similar levels of genetic diversity regardless of age or restoration status. This suggests that all plants from surrounding populations have a good opportunity to seed the new sites, as our results showed a lot of mixing between sites. Our data would indicate that new areas were colonised by individuals from the surrounding area. Success of restoration is dependent on the suitability of seed individuals to the new environment with individuals from local areas being more likely to possess adaptations to the conditions present (Gustafson et al., 2005, Bischoff et al., 2010). As saltmarsh restorations rarely incorporate any form of transplanting seed individuals must originate naturally from local areas. The resilience of saltmarsh species to maintain genetic diversity and the speed at which new population establish gives hope that we can restore saltmarshes with equivalent levels of genetic diversity if not identical genetic composition. This highlights the importance of maintaining natural marsh of good quality as even small areas have the potential to seed successful restoration sites. Our

results suggest that the greatest barriers to this success is not age or the current practice of restoration and along with previous research suggest that it is likely the non-equivalency of environmental conditions that leads to differences in the biological composition of restored saltmarsh compared to natural saltmarsh.

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Chapter 6: Discussion

6.0 Discussion

Despite inevitable changes in the climate, we lack an understanding of how many aspects of saltmarsh will respond to the resulting changes in environmental conditions. We know that flooding frequency and duration are key stressors to saltmarsh plants (Davy *et al.*, 2011) and that environmental conditions can modify species interactions (Pennings, Grant and Bertness, 2005). However, we do not have a clear understanding of how species interactions and environmental variables, such as elevation and nutrients, act in unison to influence the growth and functional traits of species, particularly under situations of increasing stress. Nor do we understand the outcomes of multi-species interactions on plant growth, partly due to the technical difficulties of managing these experiments. We are also hamstrung in our efforts as, despite its importance, we lack information on the influence of genetic identify on plant response to environmental variables, and the genetic structure of populations in natural saltmarsh environments.

Throughout this thesis, I have consistently aimed to tackle some of the barriers to understanding saltmarsh ecology. For instance, we have already identified some of the main drivers of saltmarsh plant distribution, i.e. flooding regime, species interactions, nutrient availability and dispersal. However, as a scientific community we have struggled to quantify how these effects work in combination. To tackle this problem, I employed a large multifactorial glasshouse experiment and was successful in disentangling the relative effects of species interactions and flooding. Another major barrier to our understanding was the practical difficulties in studying the effects of

multiple environmental factors in highly variable natural environments. This is compounded by the disconnect between field studies with a host of uncontrolled environmental variables, and glasshouse studies that do not effectively replicate the nuances of tidal flooding. To tackle this, I designed a new system for accurate replication of tidal flooding in a greenhouse, as well as the control of water and soil chemistry. I used this system to study the combined effects of multiple environmental variables, nutrients and flooding, on saltmarsh plant growth. Finally, there has been very little research on the role of genetics on saltmarsh plant growth and distribution. To improve this, I included genotypes as a factor within my flooding and nutrient experiment, and undertook a study to investigate how genetic populations differed between natural and restored sites, and how these populations where structured along environmental gradients. In combination with my first study, I showed that genotypes did respond differently to environmental pressures and that this difference was, at least partially, responsible for the structuring of different genetic populations. This study also provided important information from a restoration standpoint as we investigated dispersal limitation in terms of both geographic distance and age of sites.

6.1 Summary of aims and key findings

This thesis has found substantial variation in the responses of individual plants to changes in environmental conditions, including responses to the presence of other individuals. Analysis of community level effects found they were less strong than those measured at the individual level. As we progressed down levels of ecological ordering from community to between-species, within-species and finally within specific

genotypes, we found increasingly clear and repeatable patterns of response to environmental change. Individual species response was found to be context dependent on the surrounding environmental conditions and neighbouring species. Response of individual genotypes within a species was also extremely context dependent. This large amount of variation at the individual level accounts for the more muted response observed at the community level.

6.1.1 Chapter 2. Species interactions modulate the response of saltmarsh plants to flooding

Aim: To disentangle the relative effects of waterlogging and species interactions on saltmarsh plant growth and functional traits

The results showed flooding-induced waterlogging had a relatively minor impact on the functional traits of the three saltmarsh species studied and that interspecific interactions had an equivalent or greater influence on functional trait expression. I also found that whilst the direct influence of flooding was comparatively weak, it did serve to modify the effects of the interspecific interactions, changing their intensity and direction. Overall, I observed the greatest change in response of biomass and its distribution into above and below ground measures. While these responses were observed to be highly species specific, all traits were highly variable within species and treatments, and I hypothesise that this may be due to variation in the genetic composition of the individuals used. I also observed some changes at the community level with a marginal trend towards higher levels of functional traits increasing with species diversity.

6.1.2 Chapter 3. Development of a self-contained tidal inundation machine and nutrient filtering system

Aim: To design a practical laboratory system for accurate replication of tidal inundation that allows controlled manipulation of multiple nutrient regimes.

I designed and built a system that was capable of replicating real life tidal regimes using a recirculation water supply, and that successfully integrated a filtration system for nitrates and phosphates. The system accurately replicated a real life tidal system and was capable of removing nutrients well in excess of those used in previous field experiments, and levels comparable to those found in extremely eutrophied environments. The aim of the system was to allow us to test for the inter- and intra-specific and intra-genotype responses to sea level rise and different nutrient conditions (Chapter 4).

6.1.3 Chapter 4. Response of different genotypes of two saltmarsh grasses, *Puccinellia maritima* and *Festuca rubra*, to increased nutrient concentrations and sea level rise

Aim: To test if genotypes effect how plants respond (survival and growth) to increased flooding (simulated sea level rise) and to nutrient enrichment.

We hypothesised that increased nutrients would mitigate the effects of increased flooding, but found no evidence to support this and instead, responses were species-specific. We found some evidence for changes in the response of functional traits of the surviving individuals, but due to low survival overall we had relatively low replication

from which to test. Survival response was genotype-specific, with some polarised differences in survival between genotypes in the same treatment.

6.1.4 Chapter 5. Differences in genetic structure of *Puccinellia maritima* between natural saltmarshes and restored saltmarshes of different ages

Aim: To test for current differences in genetic populations of restored saltmarshes of different ages and natural saltmarsh

All of the restored sites sampled had a significantly different genetic composition of *Puccinellia maritima* to the natural marsh, and there were some differences between the restored sites, concluding that restored sites do not have the same genetic composition as natural sites. Genetic diversity also differed between sites, however this was not structured by age as the two youngest restored sites had equivalent genetic diversity to the natural site. Overall, my results suggest that age is not a defining factor in defining the genetic composition or genetic diversity in restored sites and that it is most likely differences in environmental variables that drive these changes.

6.2 Main Discussion

This thesis has consistently demonstrated high levels of variation in the response of saltmarsh plants to different stressors. It has also identified three main drivers of these responses, species interactions, environmental conditions and genetic composition. I observed significant changes in species interactions under moderate changes in environmental conditions, an effect which has been shown to define the boundaries of vegetation transitional zones (Callaway et al., 2003). When I altered environmental

conditions, I observed a change in direction and intensity of these interspecies interactions. A similar response has been found in two saltmarsh species *Spartina anglica* and *Puccinellia maritima* who do not share the same environmental niche. This is perhaps unsurprising in species that do not commonly share an environmental niche and so would be expected to be more competitive under their preferred environmental conditions (Huckle et al., 2000). However, my study species do share a common and overlapping niche and so I can conclude that either my study species were extremely sensitive to small changes in environmental conditions or there are other underlying mechanisms that have yet to be identified. The complexities of interactions make them hard to study especially when the number of species increases. However, it is important to do so as my research shows small changes in conditions can alter interactions, and more substantial alteration to environmental conditions, and therefore interactions, are likely in response to changing environmental conditions driven by climate change (Gilman et al., 2010).

The direct response of saltmarsh plants to increased flooding was relatively minor, with only some impacts on survival and effects on functional traits for some species. However, there were indirect effects of flooding through the modification of species interactions. The effects of flooding were themselves modified in the presence of increased nutrient concentrations. My results indicate that increases in flooding frequency and duration that may arise with rising sea levels may have a large impact on saltmarsh. But these effects will not simply be through modification of a species' environmental niche, as observed responses are due to a network of complex

interactions between species and other environmental conditions, such as nutrient concentrations and climate (Bertness and Ewanchuk, 2002).

I hypothesised that increased availability of nutrients (as will occur under coastal eutrophication) would offset the negative effects of increased flooding frequency and duration (as may occur with sea level rise) because previous research has found that increased nutrient availability can mitigate the impact of other stressors (Alam, 1999). However, my results did not support this hypothesis, with mortality and growth not increasing with the addition of nutrients. I did however find differences in response between different genetic clones of the species used, with some clones being more tolerant, indicating that there may be a genetic basis for tolerance. In addition, there was variation in the response of genetically indentical individuals to different environmental conditions, indicating plasticity. It is widely acknowledged that genetic composition can determine the plasticity of an individual (Ackerly et al., 2006). My results and that by other researchers on other saltmarsh species have shown that this plasticity can affect survivability and functional traits, with the potential for real world consequences on marsh species composition and ecosystem functioning (Jefferies et al., 2006; Proffitt et al., 2012; Richards et al., 2010). Furthermore, my study of the population genetics of *Puccinellia maritima* indicated that there is some environmental filtering in the populations of these species in the field, with genetic composition being correlated to an environmental gradient (elevation). In contrast, there was no indication of dispersal limitation, with the new restored saltmarsh having different genetic composition but not genetic diversity levels to natural saltmarsh.

Understanding how these results will translate to larger scales requires information on the genetic composition and geographic distribution for multiple species. However, we only have information on the geographic population structure of two species within the UK (*Puccinellia maritima* and *Triglochin maritima*) (Rouger and Jump, 2014). We know these populations differ across the country and my results show that the different genotypes that define these populations respond differently to changing environmental conditions. It is therefore likely that responses to changing climatic conditions will vary regionally. However, it is currently impossible to predict the true implications as we lack information on the genetic composition of the vast majority of species in the UK.

Due the multiple effects of species interactions, environmental conditions and genetic composition, all acting in unison within natural environments, it is extremely difficult to disentangle their relative effects. This is especially true in the case of genetic compositions where we do not have sufficient information to account for their influence. We therefore need to test for these effects within controlled laboratory conditions but we struggle to accurately and efficiently replicate natural conditions in a laboratory setting, particularly natural tidal cycles. Of the systems that have been developed, they are either extremely complicated or are not practical for most experimental use cases (Miller and Long, 2015). In this thesis, I described the development and construction of a new piece of equipment for replicating tidal cycles and controlling for nutrient concentrations in a laboratory setting. This improved upon previous systems in that it was self-contained, which allowed it to run efficiently without

continuous access to salt water. It also allowed us to tightly control water chemistry via its inbuilt filtration systems. I then demonstrated the value of this equipment by using it to investigate the response of two saltmarsh species to increased tidal inudations and nutrient concentrations, whilst controlling for genotype of the individuals. This test provided valuable information and could easily be replicated across more species not yet studied and with varying nutrient levels to build up our understanding of how an entire saltmarsh will respond to changing sea level rise and diferent nutrient addition scenarios. The design of my tidal inudation system is such that the system is extremely flexible and easy to modfiy. The filtration sysetm in paticular could be changed to study other possible stressors on saltmarsh enviroments. These could be pollutants such as oil (Hershner and Lake, 1980) or heavy metals (Williams et al., 1994), previously studied in saltmarsh. It could also be used to study other effects of climate change, such as rising dissolved CO_2 levels (Langley et al., 2013). Its use is not restricted to saltmarsh, as it could be used to study other intertidal habitats that suffer from similar pressures, such as the effects of nutrient addition in mangroves (Reef et al., 2010), or even a combination of saltmarsh and mangroves in experiments designed to understand the influences driving the changing boundaries of the environments in saltmarsh and mangrove transition zones (Saintilan et al., 2014). It could also be used to replicate tidal freshwater systems. Finally, the system is not restricted to plants, as much of the filtration system is based on hobbyist aquarium equipment and so is eminently suitable to maintain a variety of marine life and could therefore be used to investigate tidal effects on rocky shore organisms or beaches; Miller and Long (2015) used their similar equipment to investigate plant-herbivore interactions. The potential use cases are extremely varied and it is hoped this technology is adopted by the scientific community

at large, with the ultimate use-cases being designed by experts, to allow better investigation of the most important questions in their field.

Two of the biggest challenges facing saltmarsh today are restoration efforts and predicting their response to climate change. We are actively restoring saltmarsh in the UK but our current restoration efforts are limited, creating saltmarsh with non-equivalent biological (species communities) (Mossman et al., 2012) and physical (Lawrence et al., 2018) conditions to that of natural saltmarsh; I also found that the genetic composition (but not diversity) was different between natural and restored marshes. We also rely on saltmarsh to provide valuable ecosystem services and this reliance looks set to increase in the face of climate change, particularly in the light of rising sea levels. Understanding how saltmarsh respond to changing environmental conditions is critical if we are to predict the future of these habitats on which we rely.

The scientific community need to recognise the importance of genetics when designing restoration strategies. Mijangos et al. (2015) found that in 41% of reviewed studies there was no use of genetic data or theories to help guide the pre-restoration planning stages of restoration projects. In addition to this, over 59% did not gather any genetic information to help infer success, or to plan future management after restoration. Our study highlights the importance of genetic composition by showing it can be a major moderator of plant response. This will need to be considered when designing restoration projects as seed and therefore genotype availability will define the response of newly colonising individuals and the eventual functioning of the system. In systems

and species where planting regularly occur (e.g. mangroves, *Spartina* spp. in the USA), genetic composition can be manipulated to ensure genotypes have adaptations suitable for local conditions, or to maximise diversity (Seliskar et al., 2002; Granado et al., 2018). In systems such as saltmarsh in the UK, natural colonisation determines composition, as we do not conduct large-scale planting operations. I hypothesised that this natural colonisation would lead to reduced diversity, through the impact of strong founder effects in newly restored marshes. However, I did not find any evidence for these effects and even the two year old site had equivalent genetic diversity to the natural sites, and the presence of rare alleles. This suggests that there was no barrier to dispersal in this system, contrary to other research at species level (Wolters et al., 2005). I found that variation in genetic composition of restored marshes is most likely due to variations in environmental conditions. This is particularly interesting as it has already been demonstrated that restored saltmarsh have different topography and therefore different environmental conditions compared to natural saltmarsh (Lawrence et al., 2018). Establishing environmental conditions on restored marshes that are more similar to those on natural marshes may therefore improve both the species and genetic composition of the communities.

Saltmarshes provide a disproportionate amount of ecosystem services for their size compared to other environments (Zedler and Kercher, 2005). Direct measurements of ecosystem services are notoriously hard (Barbier, 2012; Cavanagh et al., 2016). We have lots of evidence that functional traits of species determine ecosystem function and can be used to estimate ecosystem service provision (de Bello et al., 2010; Lavorel, 2013). Functional traits are also a good indicator of change and can be used to monitor the

relative functioning of the environment (Doherty et al., 2011). I found functional trait responses to a range of different environmental changes including those related to the effects of climate change and increased coastal eutrophication. However, scaling these responses up to predict future changes across saltmarsh ecosystems presents a substantial challenge as we found responses were species and context dependent. My results represent an initial foundation of the type of detailed species and context specific knowledge needed to predict responses at an ecosystem level but much more research would be needed before we can achieve this.

6.3 Unanswered questions

The largest limitation of this thesis is its specificity. As I studied organisms at lower and lower levels of organisation, the power of my observations to explain effects at the overall ecosystem level decreased, as they accounted for a smaller and smaller proportion of the total number of individuals present in real world system. I have proved it necessary to study saltmarsh at these fine levels of organisation, in order to understand the intricacies of their response to environmental change. However, my results highlight the potential for a range of other specific responses of unstudied species that may also play a key role in moderating saltmarsh response as whole to environmental change.

Within this thesis I have studied five different saltmarsh species. Saltmarsh typically have poor species richness and therefore five species represents a fairly significant

proportion of the total number of species found in these habitats. However, five species is likely insufficient to make an accurate assessment of how my results may translate into real world ecosystems, particularly as I did not include all five species in each experiment. In order to improve upon the scope of this research so that we can use results to predict responses at the ecosystem level, we would need to repeat the experiments using different species. It may also be useful to increase the number of species used in such an experiment, although there are practical limitations to this. For the experiment outlined in chapter two in order to have a balanced experiment with equal replication of every possible species combination, you would require an exponentially larger number of experimental treatments for each new species added, and more individuals within each pot. These problems can be avoided by using a planting design that selects random species from a wider pool, but this will only be useful to detect community effects and my results show that interactions between specific species show a greater level of response to changing environmental conditions than others.

In addition to using a limited number of species, Chapter Four of this thesis tested responses at the genotype level and in a very specific sea level rise and nutrient addition scenario. Sea level rise will increase over time and there are a range of estimates for its relative increase under different climate change scenarios (Bamber et al., 2019). It is impossible to predict the true acceleration of climate change as we do not know how global emissions will change in the future. For the same reasons we also cannot predict future levels and composition of coastal nutrient enrichment. Whilst my results are valid for a specific scenario, they do not provide enough information for comprehensive

predictions on the future of saltmarsh, given the uncertainty surrounding our current estimates for future environmental change. In order to account for the uncertainty of future changes in environmental conditions, as well as the variation between locations, we need more experiments using the tidal inundation machine, as in Chapter Four of this thesis. These should include the use of other species, sea level rise scenarios and different concentrations and proportions of nitrate and phosphate inputs. This will allow us to predict response to a range of different sea level rise and nutrient addition scenarios and should be targeted towards most likely scenarios as the uncertainty surrounding our current predictions decrease. There is also a need for more information on the genetic composition of saltmarsh plants in the UK, so that we can apply the results of any future studies utilising different species to real world ecosystems. I have shown that there can be a significant difference in response between specific genotypes. As we do not know how these genotypes differ in real world environments, it is impossible to predict what the consequences of these differences may be. We therefore need more studies such as Rouger and Jump, (2014) to understand the genetic structure and distribution of saltmarsh species in the UK.

Finally, the tidal inundation machine I developed in Chapter Three and used in Chapter Four should allow for multiple testing of different species under different scenarios. This would broaden the breadth of the research included in this thesis and allow me to make predications across a range of likely scenarios and across larger scales of ecosystem organisation. However, the tidal inundation machine I developed is not a polished commercial product, as such it cannot be bought and must be manufactured from separately sourced components. There is also no standardised process for the

maintenance and upkeep of all the requisite components. Maintenance of the machine required very familiar knowledge of its workings and regular trouble shooting of emerging faults and this will limit its adoption by a non-specialist in its current state. In order to facilitate its use by other researchers and institutions we would need to develop a standardised construction and installation procedure. This would lower the barrier for entry, by allowing those unfamiliar with the equipment to buy a pre-packaged bundle of components. This would also help those that are familiar with the equipment to provide reliable support as we would be working with a standardised set of equipment.

6.4 Conclusion

From the results presented in this thesis, I can conclude that there is a need to consider saltmarsh plants at the individual level. The variation observed at the community level could be explained by the differences at the species and individual level. I have shown that relatively small changes in an individual's genetic makeup, subjective environmental conditions and its neighbouring individuals can dictate its response to changing environmental conditions. Climate change, as well as other anthropogenic effects, are major threats to saltmarsh habitats. If we are to accurately predict and prepare for these influences, we need more research like this thesis, which studies the response of individuals, so that we can scale up to making assessments of entire ecosystems. We also need to look back at existing studies and interpret results in the light of these findings, as average changes across the ecosystem level may be masking important changes at the species and individual level.

6.5 References

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Appendix

Appendix

Appendix 2.1

The provided macro allows for autofocus measurement of surface area of the pots. The macro only uses the base utilities present in latest version of ImageJ (ref), as such it can be ran in any unmodified copy of the software without any installation. All steps automated by the macro can be replicated manually in Image J and it is highly recommended that this be performed for a subsample of images to check that the program is working as desired. The first part of this document details how to use the macro and second details how to perform these macros function manually.

Running the macro

The macro does not include any scale settings as this would invalidate it for any images not taken at the same aspect as those used to develop the methodology. BEFORE running the macro, these settings must be input manually and be appropriate for the images used. The simplest way to do this is by following the steps below:

1. Open up an image in Image J with a scale bar taken at the same aspect ratio as those intended for use in the rest of the analysis
2. Using the line tool draw a line along the scale bar
3. In the Image J interface navigate to the following: “Analyse” – “Set scale”
4. Type the length of the scale into the known distance box tick the set global option and click ok.
5. There may be more appropriate scale settings depending on the image being used, more information on this can be found in the help files of Image J

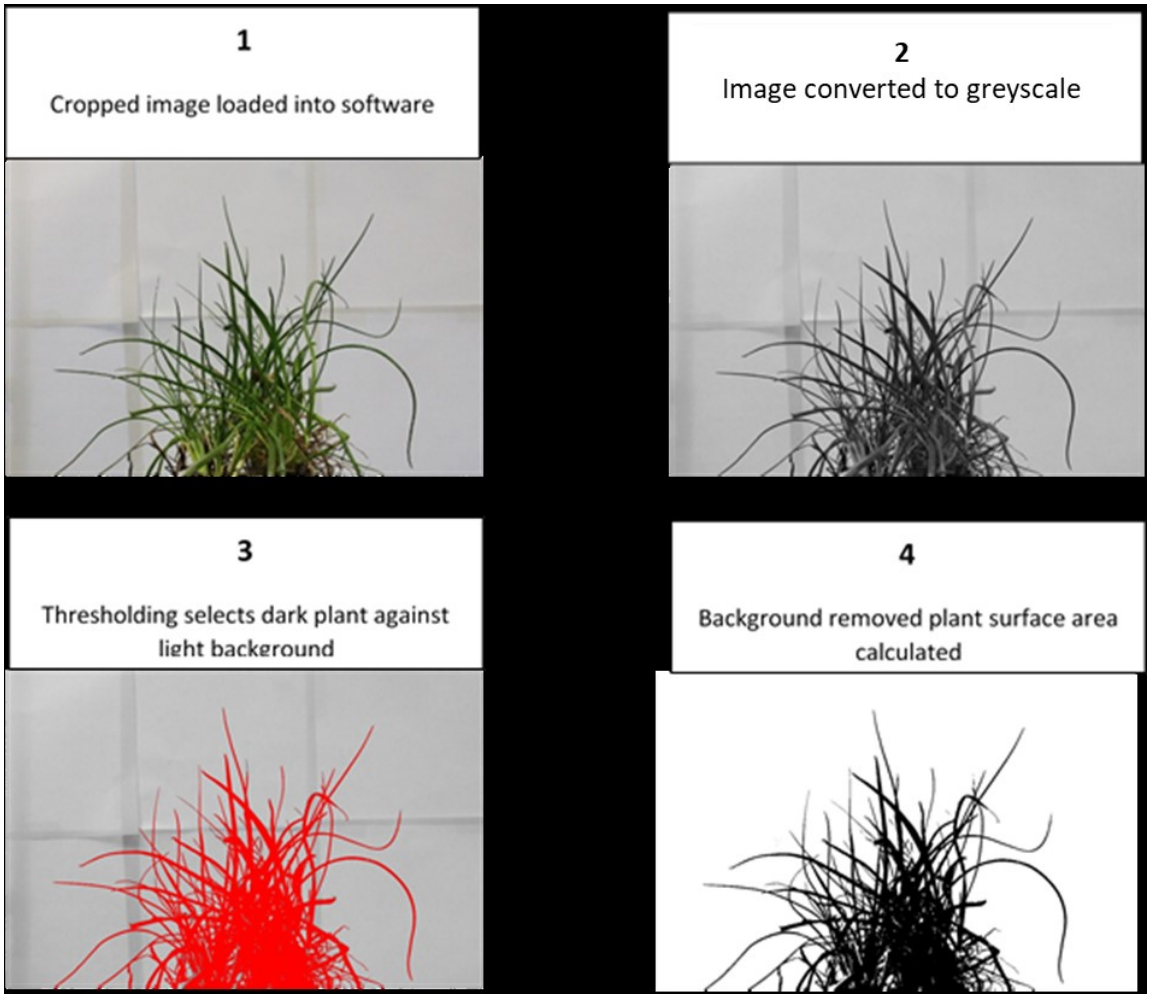
Once the scale is set to use the macro, follow these steps

1. Navigate to “plugins” – “macro”-“run”.
2. Navigate to the folder containing the macro provided with this document and select it.

3. The macro will direct to an open file explorer window if using windows or equivalent dependent on the operating system.
4. Navigate to the folder containing the images to be analysed and select the first image in the folder
5. The plugin will automatically calculate the area of the images and return a table within image j with the corresponding values.

Running the analysis manually

1. Navigate to “file”- “import image sequence” (for multiple images) “file-“ “open” (if using an individual image)
2. Select the first image in the folder or only image if using a single image
3. Image j will load in all the images excluding those that have a different resolution to the first image in the file
4. “Image”- “type” –“8bit” this converts the image to greyscale as seen in the diagram below
5. “image”-“adjust”-“ threshold” -“apply” (make sure the dark background box is unchecked)
6. This sets the default thresholding method of image j and attempts to automatically select the darker leaves of the plant against the light background. If this method does not work others are available that may be more appropriate for different images but you will be unable to the automated macro method.
7. “Analyse”- “measure”
8. If all steps are followed correctly, image j will provide a table with the area of plant in each image originally imported.


1
Cropped image loaded into software
2
Image converted to greyscale
3
Thresholding selects dark plant against light background
4
Background removed plant surface area calculated

Appendix 2.2

Table 2.2.1a Two-way ANOVA results and denoted significant differences in pair wise comparisons by species. Dark squares of pairwise comparisons denote the location of significant interaction effects.

Total Biomass

		Composition	Flooding	Composition:Flooding	Pairwise Comparisons							
	Df	3	1	3	Flooded				Unflooded			
Aster tripolium	F value	2.310	5.705	0.922	**A**	**PA**	**TA**	**PTA**	**A**	**PA**	**TA**	**PTA**
	Pr(>F)	0.078	**0.018**	0.431	a	a	a	a	a	a	a	a
	Df	3	1	3	**P**	**PA**	**PT**	**PTA**	**P**	**PA**	**PT**	**PTA**
Planatgo maritima	F value	17.651	0.518	2.677	ab	a	cd	bcd	ab	abc	d	abc
	Pr(>F)	**<0.001**	0.473	**0.048**								
	Df	3.000	1.000	3.000	**T**	**TA**	**PT**	**PTA**	**T**	**TA**	**PT**	**PTA**
Triglochin maritima	F value	7.955	2.362	0.195	abc	a	bc	abc	abc	abc	c	abc
	Pr(>F)	**<0.001**	0.126	0.899								

Above Ground Biomass

		Composition	Flooding	Composition:Flooding	Pairwise Comparisons							
	Df	3	1	3	Flooded				Unflooded			
Aster tripolium	F value	3.943	5.872	2.586	**A**	**PA**	**TA**	**PTA**	**A**	**PA**	**TA**	**PTA**
	Pr(>F)	**0.009**	**0.016**	0.054	b	a	ab	abc	b	b	ab	ab
	Df	3	1	3	**P**	**PA**	**PT**	**PTA**	**P**	**PA**	**PT**	**PTA**
Planatgo maritima	F value	8.974	1.034	1.636	ab	a	c	bc	abc	abc	c	abc
	Pr(>F)	**<0.001**	0.311	0.182								
	Df	3	1	3	**T**	**TA**	**PT**	**PTA**	**T**	**TA**	**PT**	**PTA**
Triglochin maritima	F value	6.272	3.647	0.511	ab	a	bc	ab	ab	a	ab	a
	Pr(>F)	**<0.001**	0.058	0.675								

Root mass

		Composition	Flooding	Composition:Flooding	Pairwise Comparisons							
	Df	3	1	3	Flooded				Unflooded			
Aster tripolium	F value	0.845	2.777	2.181	**A**	**PA**	**TA**	**PTA**	**A**	**PA**	**TA**	**PTA**
	Pr(>F)	0.471	0.097	0.092	a	a	a	a	a	a	a	a
	Df	3	1	3	**P**	**PA**	**PT**	**PTA**	**P**	**PA**	**PT**	**PTA**
Planatgo maritima	F value	5.303	0.077	1.610	ab	a	ab	ab	a	ab	b	ab
	Pr(>F)	**0.002**	0.782	0.188								
	Df	3	1	3	**T**	**TA**	**PT**	**PTA**	**T**	**TA**	**PT**	**PTA**
Triglochin maritima	F value	3.151	16.837	2.651	a	a	ab	**a**	ab	ab	b	**b**
	Pr(>F)	**0.026**	**<0.001**	**0.050**								

Height

		Composition	Flooding	Composition:Flooding	Pairwise Comparisons							
	Df	3	1	3	Flooded				Unflooded			
Aster tripolium	F value	2.369	0.745	2.421	**A**	**PA**	**TA**	**PTA**	**A**	**PA**	**TA**	**PTA**
	Pr(>F)	0.072	0.389	0.067	a	a	a	a	a	a	a	a
	Df	3	1	3	**P**	**PA**	**PT**	**PTA**	**P**	**PA**	**PT**	**PTA**
Planatgo maritima	F value	4.228	43.692	9.083	**b**	b	b	b	a	b	b	b
	Pr(>F)	**0.006**	**<0.001**	**<0.001**								
	Df	3	1	3	**T**	**TA**	**PT**	**PTA**	**T**	**TA**	**PT**	**PTA**
Triglochin maritima	F value	5.636	5.071	4.523	b	b	b	**b**	b	ab	b	a
	Pr(>F)	**<0.001**	**0.026**	**0.004**								

Width

		Composition	Flooding	Composition:Flooding	Pairwise Comparisons							
	Df	3	1	3	Flooded				Unflooded			
Aster tripolium	F value	2.393	0.062	1.014	**A**	**PA**	**TA**	**PTA**	**A**	**PA**	**TA**	**PTA**
	Pr(>F)	0.070	0.830	0.387	a	a	a	a	a	a	a	a
	Df	3	1	3	**P**	**PA**	**PT**	**PTA**	**P**	**PA**	**PT**	**PTA**
Planatgo maritima	F value	2.162	13.891	2.346	b	b	ab	ab	a	ab	ab	ab
	Pr(>F)	0.094	**<0.001**	0.074								
	Df	3	1	3	**T**	**TA**	**PT**	**PTA**	**T**	**TA**	**PT**	**PTA**
Triglochin maritima	F value	8.244	2.100	7.504	ab	c	ab	**bc**	bc	bc	ab	a
	Pr(>F)	**<0.001**	0.149	**<0.001**								

Number of leaves

		Composition	Flooding	Composition:Flooding	Pairwise Comparisons							
	Df	3	1	3	Flooded				Unflooded			
Aster tripolium	F value	5.233	1.457	0.334	**A**	**PA**	**TA**	**PTA**	**A**	**PA**	**TA**	**PTA**
	Pr(>F)	**0.002**	0.229	0.801	a	a	a	a	a	b	a	a
	Df	3	1	3	**P**	**PA**	**PT**	**PTA**	**P**	**PA**	**PT**	**PTA**
Planatgo maritima	F value	0.214	0.371	1.265	a	a	a	a	a	a	a	a
	Pr(>F)	0.886	0.543	0.288								
	Df	3	1	3	**T**	**TA**	**PT**	**PTA**	**T**	**TA**	**PT**	**PTA**
Triglochin maritima	F value	4.248	1.812	4.991	a	ab	ab	**b**	a	a	ab	**a**
	Pr(>F)	**0.006**	0.180	**0.002**								

Specififc leaf area

		Composition	Flooding	Composition:Flooding	Pairwise Comparisons							
	Df	3	1	3	Flooded				Unflooded			
Aster tripolium	F value	20.773	11.015	24.997	**A**	**PA**	**TA**	**PTA**	**A**	**PA**	**TA**	**PTA**
	Pr(>F)	**<0.001**	**0.001**	**<0.001**	a	**a**	a	a	a	**b**	a	a
	Df	3	1	3	**P**	**PA**	**PT**	**PTA**	**P**	**PA**	**PT**	**PTA**
Planatgo maritima	F value	14.647	5.911	5.386	a	**b**	a	a	a	**a**	a	a
	Pr(>F)	**<0.001**	**0.016**	**0.001**								
	Df	3	1	3	**T**	**TA**	**PT**	**PTA**	**T**	**TA**	**PT**	**PTA**
Triglochin maritima	F value	1.677	4.330	2.084	a	a	a	a	a	a	a	a
	Pr(>F)	0.173	**0.039**	0.104								

Table 2.2.1b Two-way ANOVA results and denoted significant differences in pair wise comparisons at pot level

Total Biomass																	
		Composition	Flooding	Composition:Flooding	**Pairwise Comparisons**												
All Pots	Df	6	1	6	**Flooded**							**Unflooded**					
	F value	12.000	0.777	0.613	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**
	Pr(>F)	**<0.001**	**0.381**	0.719	a	abc	ab	a	a	bc	ab	ab	abc	abc	ab	a	c	ab
Above Ground Biomass																	
		Composition	Flooding	Composition:Flooding	**Pairwise Comparisons**												
All Pots	Df	6	1	6	**Flooded**							**Unflooded**					
	F value	6.320	0.003	0.788	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**
	Pr(>F)	**<0.001**	**0.955**	0.582	ab	ab	ab	a	a	b	ab	ab	ab	ab	ab	a	ab	a
Root Mass																	
		Composition	Flooding	Composition:Flooding	**Pairwise Comparisons**												
All Pots	Df	6	1	6	**Flooded**							**Unflooded**					
	F value	4.271	5.868	1.713	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**
	Pr(>F)	**<0.001**	**0.018**	0.128	a	ab	a	a	a	ab	a	ab	ab	ab	ab	a	b	ab
Side on Surface Area																	
		Composition	Flooding	Composition:Flooding	**Pairwise Comparisons**												
All Pots	Df	6	1	6	**Flooded**							**Unflooded**					
	F value	6.724	0.100	2.632	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**
	Pr(>F)	**<0.001**	0.753	**0.021**	ab	abcd	ab	abcd	bcd	abc	d	abcd	a	abcd	abcd	cd	ab	abcd
Top Down Surface Area																	
		Composition	Flooding	Composition:Flooding	**Pairwise Comparisons**												
	Df	6	1	6	**Flooded**							**Unflooded**					
All Pots	F value	2.586	0.211	2.501	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**	**A**	**P**	**T**	**PA**	**TA**	**PT**	**PTA**
	Pr(>F)	**0.024**	0.647	**0.028**	a	ab	ab	b	ab	ab	ab	ab	ab	ab	b	ab	ab	ab

Table 2.2.2 Full list of all pairwise comparisons

***Aster tripolium* total biomass**							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	1479	1031	186	1.434	0.8403
A flooded	-	PTA flooded	1701	1152	186	1.476	0.8191
A flooded	-	TA flooded	217	997	186	0.218	1
A flooded	-	A unflooded	-1498	807	186	-1.856	0.5831
A flooded	-	PA unflooded	-1059	981	186	-1.08	0.9604
A flooded	-	PTA unflooded	877	1185	186	0.74	0.9956
A flooded	-	TA unflooded	361	1031	186	0.35	1
PA flooded	-	PTA flooded	222	1319	186	0.168	1
PA flooded	-	TA flooded	-1262	1185	186	-1.064	0.9633
PA flooded	-	A unflooded	-2977	1031	186	-2.887	0.0812
PA flooded	-	PA unflooded	-2538	1173	186	-2.165	0.3779
PA flooded	-	PTA unflooded	-602	1348	186	-0.447	0.9998
PA flooded	-	TA unflooded	-1118	1215	186	-0.921	0.9837
PTA flooded	-	TA flooded	-1483	1292	186	-1.148	0.945
PTA flooded	-	A unflooded	-3199	1152	186	-2.777	0.1072
PTA flooded	-	PA unflooded	-2760	1280	186	-2.156	0.3831
PTA flooded	-	PTA unflooded	-824	1442	186	-0.572	0.9992
PTA flooded	-	TA unflooded	-1340	1319	186	-1.016	0.9715
TA flooded	-	A unflooded	-1716	997	186	-1.721	0.6734
TA flooded	-	PA unflooded	-1277	1142	186	-1.118	0.9522
TA flooded	-	PTA unflooded	659	1321	186	0.499	0.9997
TA flooded	-	TA unflooded	143	1185	186	0.121	1
A unflooded	-	PA unflooded	439	981	186	0.447	0.9998
A unflooded	-	PTA unflooded	2375	1185	186	2.004	0.4819
A unflooded	-	TA unflooded	1859	1031	186	1.802	0.6193
PA unflooded	-	PTA unflooded	1936	1310	186	1.478	0.8181
PA unflooded	-	TA unflooded	1420	1173	186	1.211	0.9278
PTA unflooded	-	TA unflooded	-516	1348	186	-0.383	0.9999

***Plantago maritima* total biomass**							
Contrast			Estimate	SE	df	T ratio	P Value
PA flooded	-	P flooded	-3376.24	1399	205	-2.414	0.2402
PA flooded	-	PT Flooded	-7673.3	1569	205	-4.892	0.0001
PA flooded	-	PTA flooded	-6606.75	1746	205	-3.784	0.0049
PA flooded	-	PA unflooded	-3256.21	1569	205	-2.076	0.4341
PA flooded	-	P unflooded	-3263.72	1373	205	-2.377	0.2582
PA flooded	-	PT unflooded	-9246.91	1569	205	-5.895	<.0001
PA flooded	-	PTA unflooded	-3139.51	1817	205	-1.728	0.669
P flooded	-	PT Flooded	-4297.06	1360	205	-3.16	0.0378
P flooded	-	PTA flooded	-3230.51	1561	205	-2.069	0.4385
P flooded	-	PA unflooded	120.02	1360	205	0.088	1
P flooded	-	P unflooded	112.52	1128	205	0.1	1
P flooded	-	PT unflooded	-5870.68	1360	205	-4.317	0.0006
P flooded	-	PTA unflooded	236.73	1640	205	0.144	1
PT Flooded	-	PTA flooded	1066.55	1715	205	0.622	0.9986
PT Flooded	-	PA unflooded	4417.08	1534	205	2.879	0.0822
PT Flooded	-	P unflooded	4409.58	1333	205	3.307	0.0242
PT Flooded	-	PT unflooded	-1573.62	1534	205	-1.026	0.9701
PT Flooded	-	PTA unflooded	4533.79	1787	205	2.537	0.1861
PTA flooded	-	PA unflooded	3350.53	1715	205	1.953	0.516
PTA flooded	-	P unflooded	3343.03	1538	205	2.173	0.3723
PTA flooded	-	PT unflooded	-2640.16	1715	205	-1.539	0.7852
PTA flooded	-	PTA unflooded	3467.24	1945	205	1.783	0.6325
PA unflooded	-	P unflooded	-7.51	1333	205	-0.006	1
PA unflooded	-	PT unflooded	-5990.7	1534	205	-3.905	0.0032
PA unflooded	-	PTA unflooded	116.7	1787	205	0.065	1
P unflooded	-	PT unflooded	-5983.19	1333	205	-4.487	0.0003
P unflooded	-	PTA unflooded	124.21	1618	205	0.077	1

PT unflooded	-	PTA unflooded	6107.4	1787	205	3.417	0.0171

***Triglochin maritima* total biomass**							
Contrast			Estimate	SE	df	T ratio	P Value
PT Flooded	-	PTA flooded	3790	1681	193	2.255	0.3239
PT Flooded	-	TA flooded	5190	1496	193	3.469	0.0146
PT Flooded	-	T flooded	2432	1266	193	1.921	0.5384
PT Flooded	-	PT unflooded	-968	1478	193	-0.655	0.998
PT Flooded	-	PTA unflooded	1456	1614	193	0.902	0.9856
PT Flooded	-	TA unflooded	4238	1516	193	2.796	0.1021
PT Flooded	-	T unflooded	1682	1303	193	1.29	0.9015
PTA flooded	-	TA flooded	1400	1711	193	0.818	0.9919
PTA flooded	-	T flooded	-1358	1513	193	-0.898	0.986
PTA flooded	-	PT unflooded	-4759	1695	193	-2.808	0.0991
PTA flooded	-	PTA unflooded	-2335	1814	193	-1.287	0.9028
PTA flooded	-	TA unflooded	448	1728	193	0.259	1
PTA flooded	-	T unflooded	-2109	1545	193	-1.365	0.8719
TA flooded	-	T flooded	-2758	1306	193	-2.113	0.4106
TA flooded	-	PT unflooded	-6159	1512	193	-4.072	0.0017
TA flooded	-	PTA unflooded	-3735	1645	193	-2.27	0.3156
TA flooded	-	TA unflooded	-952	1549	193	-0.615	0.9987
TA flooded	-	T unflooded	-3508	1342	193	-2.615	0.1571
T flooded	-	PT unflooded	-3400	1285	193	-2.646	0.1463
T flooded	-	PTA unflooded	-976	1439	193	-0.678	0.9975
T flooded	-	TA unflooded	1806	1328	193	1.36	0.874
T flooded	-	T unflooded	-750	1079	193	-0.695	0.9971
PT unflooded	-	PTA unflooded	2424	1629	193	1.488	0.813
PT unflooded	-	TA unflooded	5206	1532	193	3.399	0.0183
PT unflooded	-	T unflooded	2650	1322	193	2.005	0.4814
PTA unflooded	-	TA unflooded	2782	1663	193	1.673	0.7045

PTA unflooded	-	T unflooded	226	1472	193	0.154	1
TA unflooded	-	T unflooded	-2556	1364	193	-1.875	0.5699

***Aster tripolium* above ground biomass**							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	2106.7	595	189	3.541	0.0116
A flooded	-	PTA flooded	1384	665	189	2.083	0.43
A flooded	-	TA flooded	1295.6	566	189	2.289	0.3054
A flooded	-	A unflooded	-109.2	466	189	-0.234	1
A flooded	-	PA unflooded	-192	566	189	-0.339	1
A flooded	-	PTA unflooded	1248.9	684	189	1.827	0.6025
A flooded	-	TA unflooded	364.3	575	189	0.634	0.9984
PA flooded	-	PTA flooded	-722.7	761	189	-0.95	0.9805
PA flooded	-	TA flooded	-811.1	676	189	-1.199	0.9313
PA flooded	-	A unflooded	-2216	595	189	-3.725	0.0062
PA flooded	-	PA unflooded	-2298.8	676	189	-3.399	0.0184
PA flooded	-	PTA unflooded	-857.8	777	189	-1.103	0.9554
PA flooded	-	TA unflooded	-1742.4	684	189	-2.548	0.1822
PTA flooded	-	TA flooded	-88.4	738	189	-0.12	1
PTA flooded	-	A unflooded	-1493.2	665	189	-2.247	0.3289
PTA flooded	-	PA unflooded	-1576.1	738	189	-2.135	0.3968
PTA flooded	-	PTA unflooded	-135.1	832	189	-0.162	1
PTA flooded	-	TA unflooded	-1019.7	745	189	-1.368	0.8704
TA flooded	-	A unflooded	-1404.8	566	189	-2.482	0.2098
TA flooded	-	PA unflooded	-1487.6	651	189	-2.285	0.3076
TA flooded	-	PTA unflooded	-46.7	756	189	-0.062	1
TA flooded	-	TA unflooded	-931.3	659	189	-1.413	0.8502
A unflooded	-	PA unflooded	-82.8	566	189	-0.146	1
A unflooded	-	PTA unflooded	1358.2	684	189	1.987	0.4935
A unflooded	-	TA unflooded	473.6	575	189	0.824	0.9916

PA unflooded	-	PTA unflooded	1441	756	189	1.907	0.5477
PA unflooded	-	TA unflooded	556.4	659	189	0.844	0.9902
PTA unflooded	-	TA unflooded	-884.6	762	189	-1.161	0.9419

***Plantago maritima* above ground biomass**							
Contrast			Estimate	SE	df	T ratio	P Value
PA flooded	-	P flooded	-1695	1050	208	-1.614	0.7417
PA flooded	-	PT Flooded	-4925	1192	208	-4.133	0.0013
PA flooded	-	PTA flooded	-4598	1327	208	-3.466	0.0146
PA flooded	-	PA unflooded	-2231	1192	208	-1.872	0.5716
PA flooded	-	P unflooded	-2398	1043	208	-2.3	0.2989
PA flooded	-	PT unflooded	-5154	1192	208	-4.325	0.0006
PA flooded	-	PTA unflooded	-2671	1380	208	-1.935	0.5285
P flooded	-	PT Flooded	-3230	1021	208	-3.165	0.0371
P flooded	-	PTA flooded	-2903	1175	208	-2.47	0.2141
P flooded	-	PA unflooded	-536	1021	208	-0.525	0.9995
P flooded	-	P unflooded	-703	842	208	-0.835	0.9909
P flooded	-	PT unflooded	-3459	1021	208	-3.39	0.0186
P flooded	-	PTA unflooded	-976	1236	208	-0.79	0.9935
PT Flooded	-	PTA flooded	327	1303	208	0.251	1
PT Flooded	-	PA unflooded	2694	1165	208	2.311	0.2924
PT Flooded	-	P unflooded	2526	1013	208	2.494	0.2037
PT Flooded	-	PT unflooded	-229	1165	208	-0.197	1
PT Flooded	-	PTA unflooded	2254	1358	208	1.66	0.7129
PTA flooded	-	PA unflooded	2367	1303	208	1.816	0.6096
PTA flooded	-	P unflooded	2200	1169	208	1.882	0.5647
PTA flooded	-	PT unflooded	-556	1303	208	-0.427	0.9999
PTA flooded	-	PTA unflooded	1927	1478	208	1.304	0.8965
PA unflooded	-	P unflooded	-167	1013	208	-0.165	1

PA unflooded	-	PT unflooded	-2923	1165	208	-2.508	0.1978
PA unflooded	-	PTA unflooded	-440	1358	208	-0.324	1
P unflooded	-	PT unflooded	-2756	1013	208	-2.721	0.1222
P unflooded	-	PTA unflooded	-273	1229	208	-0.222	1
PT unflooded	-	PTA unflooded	2483	1358	208	1.829	0.6011

***Triglochin maritima* above ground biomass**							
Contrast			Estimate	SE	df	T ratio	P Value
PT Flooded	-	PTA flooded	2014.2	1307	199	1.542	0.7838
PT Flooded	-	TA flooded	4186.3	1147	199	3.651	0.0079
PT Flooded	-	T flooded	1597.7	971	199	1.645	0.7223
PT Flooded	-	PT unflooded	1430	1147	199	1.247	0.9166
PT Flooded	-	PTA unflooded	4044.2	1254	199	3.225	0.0312
PT Flooded	-	TA unflooded	4066.5	1176	199	3.457	0.0151
PT Flooded	-	T unflooded	2635.2	999	199	2.639	0.1483
PTA flooded	-	TA flooded	2172.1	1328	199	1.635	0.7283
PTA flooded	-	T flooded	-416.5	1180	199	-0.353	1
PTA flooded	-	PT unflooded	-584.2	1328	199	-0.44	0.9999
PTA flooded	-	PTA unflooded	2030	1422	199	1.428	0.8434
PTA flooded	-	TA unflooded	2052.3	1354	199	1.516	0.7981
PTA flooded	-	T unflooded	621	1203	199	0.516	0.9996
TA flooded	-	T flooded	-2588.6	1000	199	-2.588	0.1665
TA flooded	-	PT unflooded	-2756.3	1171	199	-2.353	0.2707
TA flooded	-	PTA unflooded	-142.2	1276	199	-0.111	1
TA flooded	-	TA unflooded	-119.8	1200	199	-0.1	1
TA flooded	-	T unflooded	-1551.1	1027	199	-1.511	0.801
T flooded	-	PT unflooded	-167.7	1000	199	-0.168	1
T flooded	-	PTA unflooded	2446.5	1121	199	2.181	0.3675
T flooded	-	TA unflooded	2468.8	1034	199	2.388	0.2532

T flooded	-	T unflooded	1037.5	826	199	1.256	0.9137
PT unflooded	-	PTA unflooded	2614.2	1276	199	2.048	0.4525
PT unflooded	-	TA unflooded	2636.5	1200	199	2.197	0.3583
PT unflooded	-	T unflooded	1205.2	1027	199	1.174	0.9385
PTA unflooded	-	TA unflooded	22.3	1303	199	0.017	1
PTA unflooded	-	T unflooded	-1408.9	1145	199	-1.23	0.922
TA unflooded	-	T unflooded	-1431.3	1060	199	-1.351	0.8779

***Aster tripolium* rootmass**							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	-646.4	800	192	-0.808	0.9925
A flooded	-	PTA flooded	340.8	871	192	0.391	0.9999
A flooded	-	TA flooded	-1092.8	773	192	-1.413	0.8503
A flooded	-	A unflooded	-1680.5	619	192	-2.713	0.1251
A flooded	-	PA unflooded	-1056.6	740	192	-1.427	0.8435
A flooded	-	PTA unflooded	-216.5	894	192	-0.242	1
A flooded	-	TA unflooded	27.4	800	192	0.034	1
PA flooded	-	PTA flooded	987.2	1003	192	0.984	0.9763
PA flooded	-	TA flooded	-446.4	920	192	-0.485	0.9997
PA flooded	-	A unflooded	-1034.1	795	192	-1.301	0.8976
PA flooded	-	PA unflooded	-410.2	892	192	-0.46	0.9998
PA flooded	-	PTA unflooded	430	1023	192	0.42	0.9999
PA flooded	-	TA unflooded	673.8	942	192	0.715	0.9965
PTA flooded	-	TA flooded	-1433.6	982	192	-1.46	0.8276
PTA flooded	-	A unflooded	-2021.3	866	192	-2.334	0.2809
PTA flooded	-	PA unflooded	-1397.4	956	192	-1.461	0.8268
PTA flooded	-	PTA unflooded	-557.2	1080	192	-0.516	0.9996
PTA flooded	-	TA unflooded	-313.4	1003	192	-0.312	1
TA flooded	-	A unflooded	-587.7	768	192	-0.766	0.9946
TA flooded	-	PA unflooded	36.2	868	192	0.042	1
TA flooded	-	PTA unflooded	876.3	1002	192	0.874	0.988
TA flooded	-	TA unflooded	1120.2	920	192	1.218	0.9258
A unflooded	-	PA unflooded	623.9	734	192	0.85	0.9899
A unflooded	-	PTA unflooded	1464	889	192	1.647	0.7211
A unflooded	-	TA unflooded	1707.9	795	192	2.149	0.3877
PA unflooded	-	PTA unflooded	840.1	977	192	0.86	0.9891
PA unflooded	-	TA unflooded	1084	892	192	1.215	0.9266
PTA unflooded	-	TA unflooded	243.9	1023	192	0.238	1
***Plantago maritima* rootmass**							

Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	-646.4	800	192	-0.808	0.9925
A flooded	-	PTA flooded	340.8	871	192	0.391	0.9999
A flooded	-	TA flooded	-1092.8	773	192	-1.413	0.8503
A flooded	-	A unflooded	-1680.5	619	192	-2.713	0.1251
A flooded	-	PA unflooded	-1056.6	740	192	-1.427	0.8435
A flooded	-	PTA unflooded	-216.5	894	192	-0.242	1
A flooded	-	TA unflooded	27.4	800	192	0.034	1
PA flooded	-	PTA flooded	987.2	1003	192	0.984	0.9763
PA flooded	-	TA flooded	-446.4	920	192	-0.485	0.9997
PA flooded	-	A unflooded	-1034.1	795	192	-1.301	0.8976
PA flooded	-	PA unflooded	-410.2	892	192	-0.46	0.9998
PA flooded	-	PTA unflooded	430	1023	192	0.42	0.9999
PA flooded	-	TA unflooded	673.8	942	192	0.715	0.9965
PTA flooded	-	TA flooded	-1433.6	982	192	-1.46	0.8276
PTA flooded	-	A unflooded	-2021.3	866	192	-2.334	0.2809
PTA flooded	-	PA unflooded	-1397.4	956	192	-1.461	0.8268
PTA flooded	-	PTA unflooded	-557.2	1080	192	-0.516	0.9996
PTA flooded	-	TA unflooded	-313.4	1003	192	-0.312	1
TA flooded	-	A unflooded	-587.7	768	192	-0.766	0.9946
TA flooded	-	PA unflooded	36.2	868	192	0.042	1
TA flooded	-	PTA unflooded	876.3	1002	192	0.874	0.988
TA flooded	-	TA unflooded	1120.2	920	192	1.218	0.9258
A unflooded	-	PA unflooded	623.9	734	192	0.85	0.9899
A unflooded	-	PTA unflooded	1464	889	192	1.647	0.7211
A unflooded	-	TA unflooded	1707.9	795	192	2.149	0.3877
PA unflooded	-	PTA unflooded	840.1	977	192	0.86	0.9891
PA unflooded	-	TA unflooded	1084	892	192	1.215	0.9266
PTA unflooded	-	TA unflooded	243.9	1023	192	0.238	1

***Triglochin maritima* rootmass**

Contrast			Estimate	SE	df	T ratio	P Value
PT Flooded	-	PTA flooded	1839.7	996	205	1.848	0.5884
PT Flooded	-	TA flooded	1220.3	902	205	1.353	0.877
PT Flooded	-	T flooded	514.8	784	205	0.657	0.9979
PT Flooded	-	PT unflooded	-2102.7	902	205	-2.331	0.2819
PT Flooded	-	PTA unflooded	-2442.5	996	205	-2.453	0.222
PT Flooded	-	TA unflooded	556.3	892	205	0.623	0.9985
PT Flooded	-	T unflooded	-637.3	790	205	-0.807	0.9926
PTA flooded	-	TA flooded	-619.4	996	205	-0.622	0.9985
PTA flooded	-	T flooded	-1325	890	205	-1.488	0.813
PTA flooded	-	PT unflooded	-3942.4	996	205	-3.959	0.0026
PTA flooded	-	PTA unflooded	-4282.3	1081	205	-3.96	0.0026
PTA flooded	-	TA unflooded	-1283.4	987	205	-1.3	0.898
PTA flooded	-	T unflooded	-2477	896	205	-2.765	0.1097
TA flooded	-	T flooded	-705.6	784	205	-0.9	0.9858
TA flooded	-	PT unflooded	-3323	902	205	-3.684	0.007
TA flooded	-	PTA unflooded	-3662.9	996	205	-3.679	0.0071
TA flooded	-	TA unflooded	-664	892	205	-0.744	0.9955
TA flooded	-	T unflooded	-1857.6	790	205	-2.351	0.2716
T flooded	-	PT unflooded	-2617.4	784	205	-3.339	0.022
T flooded	-	PTA unflooded	-2957.3	890	205	-3.322	0.0231
T flooded	-	TA unflooded	41.6	773	205	0.054	1
T flooded	-	T unflooded	-1152	652	205	-1.766	0.6436
PT unflooded	-	PTA unflooded	-339.9	996	205	-0.341	1
PT unflooded	-	TA unflooded	2659	892	205	2.979	0.063
PT unflooded	-	T unflooded	1465.4	790	205	1.855	0.5836
PTA unflooded	-	TA unflooded	2998.9	987	205	3.038	0.0536
PTA unflooded	-	T unflooded	1805.3	896	205	2.016	0.474
TA unflooded	-	T unflooded	-1193.6	779	205	-1.532	0.7895

Aster tripolium Height							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	33.31	12.9	194	2.58	0.1698
A flooded	-	PTA flooded	41.48	14.3	194	2.899	0.0783
A flooded	-	TA flooded	11.72	12.5	194	0.937	0.982
A flooded	-	A unflooded	6.61	10.2	194	0.646	0.9981
A flooded	-	PA unflooded	2.27	12.2	194	0.187	1
A flooded	-	PTA unflooded	13.6	14.7	194	0.926	0.9832
A flooded	-	TA unflooded	22.06	12.9	194	1.709	0.6817
PA flooded	-	PTA flooded	8.17	16.3	194	0.501	0.9996
PA flooded	-	TA flooded	-21.59	14.7	194	-1.465	0.8251
PA flooded	-	A unflooded	-26.7	12.9	194	-2.075	0.4345
PA flooded	-	PA unflooded	-31.04	14.4	194	-2.149	0.3877
PA flooded	-	PTA unflooded	-19.71	16.6	194	-1.186	0.9352
PA flooded	-	TA unflooded	-11.25	15.1	194	-0.746	0.9954
PTA flooded	-	TA flooded	-29.76	16	194	-1.863	0.5782
PTA flooded	-	A unflooded	-34.87	14.3	194	-2.444	0.2262
PTA flooded	-	PA unflooded	-39.21	15.7	194	-2.497	0.2031
PTA flooded	-	PTA unflooded	-27.88	17.7	194	-1.573	0.7661
PTA flooded	-	TA unflooded	-19.42	16.3	194	-1.191	0.9336
TA flooded	-	A unflooded	-5.11	12.5	194	-0.41	0.9999
TA flooded	-	PA unflooded	-9.45	14.1	194	-0.671	0.9976
TA flooded	-	PTA unflooded	1.88	16.3	194	0.115	1
TA flooded	-	TA unflooded	10.34	14.7	194	0.702	0.9969
A unflooded	-	PA unflooded	-4.34	12.1	194	-0.358	1
A unflooded	-	PTA unflooded	6.99	14.6	194	0.477	0.9997
A unflooded	-	TA unflooded	15.45	12.9	194	1.201	0.9308
PA unflooded	-	PTA unflooded	11.33	16	194	0.706	0.9967
PA unflooded	-	TA unflooded	19.79	14.4	194	1.37	0.8697
PTA unflooded	-	TA unflooded	8.46	16.6	194	0.509	0.9996

***Plantago maritima*Height**							
Contrast			Estimate	SE	df	T ratio	P Value
PA flooded	-	P flooded	-6.28	13.4	214	-0.468	0.9998
PA flooded	-	PT Flooded	9.38	15.4	214	0.607	0.9988
PA flooded	-	PTA flooded	29.4	17.3	214	1.703	0.6855
PA flooded	-	PA unflooded	13.75	15.4	214	0.89	0.9867
PA flooded	-	P unflooded	84.57	13.4	214	6.302	<.0001
PA flooded	-	PT unflooded	32.29	15.4	214	2.091	0.4239
PA flooded	-	PTA unflooded	36.77	17.3	214	2.13	0.3992
P flooded	-	PT Flooded	15.66	13.4	214	1.167	0.9405
P flooded	-	PTA flooded	35.68	15.5	214	2.304	0.2961
P flooded	-	PA unflooded	20.03	13.4	214	1.493	0.8107
P flooded	-	P unflooded	90.85	11	214	8.234	<.0001
P flooded	-	PT unflooded	38.57	13.4	214	2.874	0.0831
P flooded	-	PTA unflooded	43.05	15.5	214	2.781	0.1054
PT Flooded	-	PTA flooded	20.02	17.3	214	1.16	0.9423
PT Flooded	-	PA unflooded	4.38	15.4	214	0.283	1
PT Flooded	-	P unflooded	75.2	13.4	214	5.603	<.0001
PT Flooded	-	PT unflooded	22.92	15.4	214	1.484	0.8152
PT Flooded	-	PTA unflooded	27.4	17.3	214	1.587	0.7577
PTA flooded	-	PA unflooded	-15.65	17.3	214	-0.906	0.9852
PTA flooded	-	P unflooded	55.17	15.5	214	3.564	0.0105
PTA flooded	-	PT unflooded	2.9	17.3	214	0.168	1
PTA flooded	-	PTA unflooded	7.38	18.9	214	0.39	0.9999
PA unflooded	-	P unflooded	70.82	13.4	214	5.277	<.0001
PA unflooded	-	PT unflooded	18.54	15.4	214	1.201	0.931
PA unflooded	-	PTA unflooded	23.02	17.3	214	1.333	0.8851
P unflooded	-	PT unflooded	-52.28	13.4	214	-3.896	0.0032
P unflooded	-	PTA unflooded	-47.8	15.5	214	-3.087	0.0464
PT unflooded	-	PTA unflooded	4.48	17.3	214	0.259	1

***Triglochin maritima*Height**							
Contrast			Estimate	SE	df	T ratio	P Value
PT Flooded	-	PTA flooded	-10.2917	24.2	211	-0.425	0.9999
PT Flooded	-	TA flooded	-13.6553	22.1	211	-0.617	0.9986
PT Flooded	-	T flooded	-24.4792	18.7	211	-1.306	0.8957
PT Flooded	-	PT unflooded	-13.75	21.6	211	-0.635	0.9983
PT Flooded	-	PTA unflooded	90.6646	24.2	211	3.748	0.0056
PT Flooded	-	TA unflooded	21.1174	22.1	211	0.955	0.9801
PT Flooded	-	T unflooded	-20.0044	18.8	211	-1.064	0.9635
PTA flooded	-	TA flooded	-3.3636	24.6	211	-0.137	1
PTA flooded	-	T flooded	-14.1875	21.6	211	-0.656	0.998
PTA flooded	-	PT unflooded	-3.4583	24.2	211	-0.143	1
PTA flooded	-	PTA unflooded	100.9562	26.5	211	3.81	0.0045
PTA flooded	-	TA unflooded	31.4091	24.6	211	1.275	0.907
PTA flooded	-	T unflooded	-9.7128	21.7	211	-0.448	0.9998
TA flooded	-	T flooded	-10.8239	19.3	211	-0.561	0.9993
TA flooded	-	PT unflooded	-0.0947	22.1	211	-0.004	1
TA flooded	-	PTA unflooded	104.3199	24.6	211	4.236	0.0009
TA flooded	-	TA unflooded	34.7727	22.6	211	1.539	0.7856
TA flooded	-	T unflooded	-6.3491	19.4	211	-0.328	1
T flooded	-	PT unflooded	10.7292	18.7	211	0.573	0.9992
T flooded	-	PTA unflooded	115.1437	21.6	211	5.321	<.0001
T flooded	-	TA unflooded	45.5966	19.3	211	2.363	0.2654
T flooded	-	T unflooded	4.4747	15.4	211	0.291	1
PT unflooded	-	PTA unflooded	104.4146	24.2	211	4.316	0.0006
PT unflooded	-	TA unflooded	34.8674	22.1	211	1.576	0.7641
PT unflooded	-	T unflooded	-6.2544	18.8	211	-0.333	1
PTA unflooded	-	TA unflooded	-69.5472	24.6	211	-2.824	0.0946
PTA unflooded	-	T unflooded	-110.669	21.7	211	-5.101	<.0001

TA unflooded	-	T unflooded	-41.1219	19.4	211	-2.124	0.4031

***Aster tripolium* width**							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	36.82	16.5	194	2.235	0.3356
A flooded	-	PTA flooded	47.14	18.3	194	2.583	0.1687
A flooded	-	TA flooded	22.25	16	194	1.395	0.8588
A flooded	-	A unflooded	14.87	13.1	194	1.139	0.9473
A flooded	-	PA unflooded	24.9	15.5	194	1.606	0.7463
A flooded	-	PTA unflooded	26.53	18.7	194	1.417	0.8486
A flooded	-	TA unflooded	8.32	16.5	194	0.505	0.9996
PA flooded	-	PTA flooded	10.32	20.8	194	0.496	0.9997
PA flooded	-	TA flooded	-14.57	18.8	194	-0.775	0.9942
PA flooded	-	A unflooded	-21.95	16.4	194	-1.338	0.8833
PA flooded	-	PA unflooded	-11.92	18.4	194	-0.647	0.9981
PA flooded	-	PTA unflooded	-10.29	21.2	194	-0.485	0.9997
PA flooded	-	TA unflooded	-28.5	19.2	194	-1.481	0.8169
PTA flooded	-	TA flooded	-24.88	20.4	194	-1.221	0.9248
PTA flooded	-	A unflooded	-32.27	18.2	194	-1.773	0.6388
PTA flooded	-	PA unflooded	-22.23	20	194	-1.11	0.9541
PTA flooded	-	PTA unflooded	-20.6	22.6	194	-0.911	0.9847
PTA flooded	-	TA unflooded	-38.82	20.8	194	-1.867	0.5751
TA flooded	-	A unflooded	-7.39	15.9	194	-0.465	0.9998
TA flooded	-	PA unflooded	2.65	18	194	0.148	1
TA flooded	-	PTA unflooded	4.28	20.8	194	0.206	1
TA flooded	-	TA unflooded	-13.93	18.8	194	-0.741	0.9956
A unflooded	-	PA unflooded	10.04	15.4	194	0.65	0.9981
A unflooded	-	PTA unflooded	11.67	18.7	194	0.625	0.9985
A unflooded	-	TA unflooded	-6.55	16.4	194	-0.399	0.9999
PA unflooded	-	PTA unflooded	1.63	20.5	194	0.08	1
PA unflooded	-	TA unflooded	-16.58	18.4	194	-0.9	0.9858

PTA unflooded	-	TA unflooded	-18.21	21.2	194	-0.859	0.9892

***Plantago maritima*width**							
Contrast			Estimate	SE	df	T ratio	P Value
PA flooded	-	P flooded	32.54	22.5	214	1.445	0.8351
PA flooded	-	PT Flooded	46.46	25.9	214	1.793	0.6257
PA flooded	-	PTA flooded	48.85	29	214	1.686	0.6962
PA flooded	-	PA unflooded	59.17	25.9	214	2.283	0.3078
PA flooded	-	P unflooded	105.63	22.5	214	4.69	0.0001
PA flooded	-	PT unflooded	55.21	25.9	214	2.13	0.3989
PA flooded	-	PTA unflooded	43.85	29	214	1.514	0.7994
P flooded	-	PT Flooded	13.91	22.5	214	0.618	0.9986
P flooded	-	PTA flooded	16.31	26	214	0.628	0.9985
P flooded	-	PA unflooded	26.62	22.5	214	1.182	0.9363
P flooded	-	P unflooded	73.09	18.5	214	3.947	0.0027
P flooded	-	PT unflooded	22.66	22.5	214	1.006	0.9731
P flooded	-	PTA unflooded	11.31	26	214	0.435	0.9999
PT Flooded	-	PTA flooded	2.4	29	214	0.083	1
PT Flooded	-	PA unflooded	12.71	25.9	214	0.49	0.9997
PT Flooded	-	P unflooded	59.17	22.5	214	2.627	0.152
PT Flooded	-	PT unflooded	8.75	25.9	214	0.338	1
PT Flooded	-	PTA unflooded	-2.6	29	214	-0.09	1
PTA flooded	-	PA unflooded	10.31	29	214	0.356	1
PTA flooded	-	P unflooded	56.78	26	214	2.185	0.3649
PTA flooded	-	PT unflooded	6.35	29	214	0.219	1
PTA flooded	-	PTA unflooded	-5	31.7	214	-0.158	1
PA unflooded	-	P unflooded	46.46	22.5	214	2.063	0.4424
PA unflooded	-	PT unflooded	-3.96	25.9	214	-0.153	1

PA unflooded	-	PTA unflooded	-15.31	29	214	-0.529	0.9995
P unflooded	-	PT unflooded	-50.42	22.5	214	-2.239	0.333
P unflooded	-	PTA unflooded	-61.78	26	214	-2.378	0.2578
PT unflooded	-	PTA unflooded	-11.35	29	214	-0.392	0.9999

***Triglochin maritima*width**							
Contrast			Estimate	SE	df	T ratio	P Value
PT Flooded	-	PTA flooded	-86.81	31	211	-2.801	0.1003
PT Flooded	-	TA flooded	-131.59	28.3	211	-4.642	0.0002
PT Flooded	-	T flooded	-36.46	24	211	-1.518	0.7968
PT Flooded	-	PT unflooded	-17.92	27.7	211	-0.646	0.9981
PT Flooded	-	PTA unflooded	46.37	31	211	1.496	0.809
PT Flooded	-	TA unflooded	-71.82	28.3	211	-2.534	0.1873
PT Flooded	-	T unflooded	-64.73	24.1	211	-2.687	0.1325
PTA flooded	-	TA flooded	-44.78	31.6	211	-1.419	0.8476
PTA flooded	-	T flooded	50.35	27.7	211	1.816	0.6098
PTA flooded	-	PT unflooded	68.9	31	211	2.223	0.3425
PTA flooded	-	PTA unflooded	133.18	34	211	3.922	0.0029
PTA flooded	-	TA unflooded	14.99	31.6	211	0.475	0.9998
PTA flooded	-	T unflooded	22.08	27.8	211	0.794	0.9933
TA flooded	-	T flooded	95.13	24.7	211	3.847	0.0039
TA flooded	-	PT unflooded	113.67	28.3	211	4.01	0.0021
TA flooded	-	PTA unflooded	177.96	31.6	211	5.64	<.0001
TA flooded	-	TA unflooded	59.77	29	211	2.064	0.4416
TA flooded	-	T unflooded	66.86	24.8	211	2.695	0.1299
T flooded	-	PT unflooded	18.54	24	211	0.772	0.9943
T flooded	-	PTA unflooded	82.83	27.7	211	2.988	0.0614
T flooded	-	TA unflooded	-35.36	24.7	211	-1.43	0.8424
T flooded	-	T unflooded	-28.28	19.7	211	-1.435	0.8401
PT unflooded	-	PTA unflooded	64.29	31	211	2.074	0.4352

PT unflooded	-	TA unflooded	-53.9	28.3	211	-1.901	0.5515
PT unflooded	-	T unflooded	-46.82	24.1	211	-1.943	0.523
PTA unflooded	-	TA unflooded	-118.19	31.6	211	-3.745	0.0056
PTA unflooded	-	T unflooded	-111.1	27.8	211	-3.997	0.0022
TA unflooded	-	T unflooded	7.08	24.8	211	0.286	1

***Aster tripolium* leaf number**							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	7.601	3.69	194	2.062	0.4431
A flooded	-	PTA flooded	8.318	4.08	194	2.037	0.4599
A flooded	-	TA flooded	-0.485	3.57	194	-0.136	1
A flooded	-	A unflooded	-2.167	2.92	194	-0.742	0.9956
A flooded	-	PA unflooded	7.443	3.47	194	2.145	0.39
A flooded	-	PTA unflooded	1.723	4.19	194	0.411	0.9999
A flooded	-	TA unflooded	-2.399	3.69	194	-0.651	0.9981
PA flooded	-	PTA flooded	0.717	4.65	194	0.154	1
PA flooded	-	TA flooded	-8.086	4.21	194	-1.922	0.5375
PA flooded	-	A unflooded	-9.768	3.67	194	-2.66	0.1416
PA flooded	-	PA unflooded	-0.158	4.12	194	-0.038	1
PA flooded	-	PTA unflooded	-5.879	4.75	194	-1.239	0.9192
PA flooded	-	TA unflooded	-10	4.31	194	-2.322	0.287
PTA flooded	-	TA flooded	-8.803	4.56	194	-1.931	0.5317
PTA flooded	-	A unflooded	-10.485	4.07	194	-2.575	0.1715
PTA flooded	-	PA unflooded	-0.875	4.48	194	-0.195	1
PTA flooded	-	PTA unflooded	-6.595	5.06	194	-1.303	0.8967
PTA flooded	-	TA unflooded	-10.717	4.65	194	-2.304	0.2968
TA flooded	-	A unflooded	-1.682	3.56	194	-0.473	0.9998
TA flooded	-	PA unflooded	7.928	4.02	194	1.972	0.5032
TA flooded	-	PTA unflooded	2.208	4.66	194	0.474	0.9998
TA flooded	-	TA unflooded	-1.914	4.21	194	-0.455	0.9998
A unflooded	-	PA unflooded	9.61	3.46	194	2.781	0.1059

A unflooded	-	PTA unflooded	3.89	4.18	194	0.931	0.9827
A unflooded	-	TA unflooded	-0.232	3.67	194	-0.063	1
PA unflooded	-	PTA unflooded	-5.72	4.58	194	-1.249	0.9159
PA unflooded	-	TA unflooded	-9.842	4.12	194	-2.387	0.2536
PTA unflooded	-	TA unflooded	-4.121	4.75	194	-0.869	0.9885

***Plantago maritima* leaf number**							
Contrast			Estimate	SE	df	T ratio	P Value
PA flooded	-	P flooded	1.414	5.03	214	0.281	1
PA flooded	-	PT Flooded	2.125	5.79	214	0.367	1
PA flooded	-	PTA flooded	9.354	6.47	214	1.445	0.8351
PA flooded	-	PA unflooded	8.75	5.79	214	1.511	0.8008
PA flooded	-	P unflooded	2.84	5.03	214	0.564	0.9992
PA flooded	-	PT unflooded	4.208	5.79	214	0.727	0.9961
PA flooded	-	PTA unflooded	0.292	6.47	214	0.045	1
P flooded	-	PT Flooded	0.711	5.03	214	0.141	1
P flooded	-	PTA flooded	7.94	5.81	214	1.368	0.8709
P flooded	-	PA unflooded	7.336	5.03	214	1.458	0.8287
P flooded	-	P unflooded	1.426	4.14	214	0.345	1
P flooded	-	PT unflooded	2.794	5.03	214	0.555	0.9993
P flooded	-	PTA unflooded	-1.122	5.81	214	-0.193	1
PT Flooded	-	PTA flooded	7.229	6.47	214	1.117	0.9526
PT Flooded	-	PA unflooded	6.625	5.79	214	1.144	0.9462
PT Flooded	-	P unflooded	0.715	5.03	214	0.142	1
PT Flooded	-	PT unflooded	2.083	5.79	214	0.36	1
PT Flooded	-	PTA unflooded	-1.833	6.47	214	-0.283	1
PTA flooded	-	PA unflooded	-0.604	6.47	214	-0.093	1
PTA flooded	-	P unflooded	-6.515	5.81	214	-1.122	0.9514
PTA flooded	-	PT unflooded	-5.146	6.47	214	-0.795	0.9932

PTA flooded	-	PTA unflooded	-9.062	7.09	214	-1.278	0.9061
PA unflooded	-	P unflooded	-5.91	5.03	214	-1.174	0.9384
PA unflooded	-	PT unflooded	-4.542	5.79	214	-0.784	0.9938
PA unflooded	-	PTA unflooded	-8.458	6.47	214	-1.307	0.8956
P unflooded	-	PT unflooded	1.369	5.03	214	0.272	1
P unflooded	-	PTA unflooded	-2.548	5.81	214	-0.439	0.9999
PT unflooded	-	PTA unflooded	-3.917	6.47	214	-0.605	0.9988

***Triglochin maritima* leaf number**							
Contrast			Estimate	SE	df	T ratio	P Value
PT Flooded	-	PTA flooded	-12.542	4.43	211	-2.83	0.0931
PT Flooded	-	TA flooded	-2.951	4.05	211	-0.728	0.9961
PT Flooded	-	T flooded	5.854	3.43	211	1.706	0.6836
PT Flooded	-	PT unflooded	-2.667	3.96	211	-0.673	0.9976
PT Flooded	-	PTA unflooded	5.208	4.43	211	1.175	0.9381
PT Flooded	-	TA unflooded	3.595	4.05	211	0.887	0.987
PT Flooded	-	T unflooded	3.948	3.44	211	1.146	0.9457
PTA flooded	-	TA flooded	9.591	4.51	211	2.126	0.4016
PTA flooded	-	T flooded	18.396	3.96	211	4.642	0.0002
PTA flooded	-	PT unflooded	9.875	4.43	211	2.229	0.339
PTA flooded	-	PTA unflooded	17.75	4.85	211	3.657	0.0076
PTA flooded	-	TA unflooded	16.136	4.51	211	3.577	0.01
PTA flooded	-	T unflooded	16.489	3.97	211	4.15	0.0012
TA flooded	-	T flooded	8.805	3.53	211	2.491	0.205
TA flooded	-	PT unflooded	0.284	4.05	211	0.07	1
TA flooded	-	PTA unflooded	8.159	4.51	211	1.809	0.6148
TA flooded	-	TA unflooded	6.545	4.14	211	1.581	0.761
TA flooded	-	T unflooded	6.898	3.55	211	1.945	0.5216
T flooded	-	PT unflooded	-8.521	3.43	211	-2.483	0.2087
T flooded	-	PTA unflooded	-0.646	3.96	211	-0.163	1

T flooded	-	TA unflooded	-2.259	3.53	211	-0.639	0.9983
T flooded	-	T unflooded	-1.906	2.82	211	-0.677	0.9975
PT unflooded	-	PTA unflooded	7.875	4.43	211	1.777	0.6361
PT unflooded	-	TA unflooded	6.261	4.05	211	1.545	0.7819
PT unflooded	-	T unflooded	6.614	3.44	211	1.92	0.5385
PTA unflooded	-	TA unflooded	-1.614	4.51	211	-0.358	1
PTA unflooded	-	T unflooded	-1.261	3.97	211	-0.317	1
TA unflooded	-	T unflooded	0.353	3.55	211	0.1	1

Aster tripolium **specific leaf area**							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	-55.294	5.03	178	-10.993	<.0001
A flooded	-	PTA flooded	-1.021	6.17	178	-0.165	1
A flooded	-	TA flooded	-2.211	4.72	178	-0.469	0.9998
A flooded	-	A unflooded	-6.191	3.99	178	-1.553	0.7771
A flooded	-	PA unflooded	-1.187	5.03	178	-0.236	1
A flooded	-	PTA unflooded	1.679	6.69	178	0.251	1
A flooded	-	TA unflooded	-0.805	4.86	178	-0.166	1
PA flooded	-	PTA flooded	54.273	6.92	178	7.845	<.0001
PA flooded	-	TA flooded	53.083	5.66	178	9.377	<.0001
PA flooded	-	A unflooded	49.103	5.07	178	9.69	<.0001
PA flooded	-	PA unflooded	54.107	5.92	178	9.133	<.0001
PA flooded	-	PTA unflooded	56.973	7.39	178	7.711	<.0001
PA flooded	-	TA unflooded	54.489	5.78	178	9.425	<.0001
PTA flooded	-	TA flooded	-1.19	6.69	178	-0.178	1
PTA flooded	-	A unflooded	-5.17	6.2	178	-0.834	0.9909
PTA flooded	-	PA unflooded	-0.166	6.92	178	-0.024	1
PTA flooded	-	PTA unflooded	2.7	8.21	178	0.329	1
PTA flooded	-	TA unflooded	0.216	6.8	178	0.032	1

TA flooded	-	A unflooded	-3.98	4.76	178	-0.837	0.9907
TA flooded	-	PA unflooded	1.025	5.66	178	0.181	1
TA flooded	-	PTA unflooded	3.89	7.18	178	0.542	0.9994
TA flooded	-	TA unflooded	1.406	5.51	178	0.255	1
A unflooded	-	PA unflooded	5.005	5.07	178	0.988	0.9757
A unflooded	-	PTA unflooded	7.87	6.72	178	1.171	0.9391
A unflooded	-	TA unflooded	5.386	4.9	178	1.099	0.9563
PA unflooded	-	PTA unflooded	2.865	7.39	178	0.388	0.9999
PA unflooded	-	TA unflooded	0.381	5.78	178	0.066	1
PTA unflooded	-	TA unflooded	-2.484	7.27	178	-0.341	1

***Plantago maritima* specific leaf area**							
Contrast			Estimate	SE	df	T ratio	P Value
PA flooded	-	P flooded	21.46937	3.18	189	6.748	<.0001
PA flooded	-	PT Flooded	22.42084	3.64	189	6.151	<.0001
PA flooded	-	PTA flooded	22.88601	4.05	189	5.654	<.0001
PA flooded	-	PA unflooded	16.8974	3.61	189	4.687	0.0001
PA flooded	-	P unflooded	21.96752	3.19	189	6.876	<.0001
PA flooded	-	PT unflooded	23.30146	3.79	189	6.155	<.0001
PA flooded	-	PTA unflooded	22.41872	4.23	189	5.301	<.0001
P flooded	-	PT Flooded	0.95147	3.18	189	0.299	1
P flooded	-	PTA flooded	1.41664	3.64	189	0.39	0.9999
P flooded	-	PA unflooded	-4.57197	3.14	189	-1.458	0.8285
P flooded	-	P unflooded	0.49815	2.65	189	0.188	1
P flooded	-	PT unflooded	1.83208	3.34	189	0.548	0.9994
P flooded	-	PTA unflooded	0.94935	3.84	189	0.247	1
PT Flooded	-	PTA flooded	0.46517	4.05	189	0.115	1
PT Flooded	-	PA unflooded	-5.52344	3.61	189	-1.532	0.7891
PT Flooded	-	P unflooded	-0.45332	3.19	189	-0.142	1

PT Flooded	-	PT unflooded	0.88061	3.79	189	0.233	1
PT Flooded	-	PTA unflooded	-0.00212	4.23	189	-0.001	1
PTA flooded	-	PA unflooded	-5.98861	4.01	189	-1.493	0.8106
PTA flooded	-	P unflooded	-0.91849	3.65	189	-0.252	1
PTA flooded	-	PT unflooded	0.41545	4.18	189	0.099	1
PTA flooded	-	PTA unflooded	-0.46729	4.58	189	-0.102	1
PA unflooded	-	P unflooded	5.07012	3.15	189	1.61	0.7439
PA unflooded	-	PT unflooded	6.40405	3.75	189	1.709	0.6816
PA unflooded	-	PTA unflooded	5.52132	4.19	189	1.316	0.8918
P unflooded	-	PT unflooded	1.33393	3.36	189	0.398	0.9999
P unflooded	-	PTA unflooded	0.4512	3.85	189	0.117	1
PT unflooded	-	PTA unflooded	-0.88274	4.35	189	-0.203	1

***Triglochin maritima* specific leaf area**

Contrast			Estimate	SE	df	T ratio	P Value
PT Flooded	-	PTA flooded	1.2205	4.21	188	0.29	1
PT Flooded	-	TA flooded	-3.2033	3.59	188	-0.893	0.9864
PT Flooded	-	T flooded	0.0191	3.04	188	0.006	1
PT Flooded	-	PT unflooded	-6.7256	3.55	188	-1.896	0.5555
PT Flooded	-	PTA unflooded	-11.4682	4.33	188	-2.65	0.1451
PT Flooded	-	TA unflooded	-4.3775	3.55	188	-1.234	0.9208
PT Flooded	-	T unflooded	-0.503	3.15	188	-0.16	1
PTA flooded	-	TA flooded	-4.4238	4.21	188	-1.051	0.9657
PTA flooded	-	T flooded	-1.2014	3.75	188	-0.32	1
PTA flooded	-	PT unflooded	-7.9461	4.17	188	-1.904	0.5498
PTA flooded	-	PTA unflooded	-12.6887	4.85	188	-2.614	0.1574
PTA flooded	-	TA unflooded	-5.598	4.17	188	-1.341	0.8817
PTA flooded	-	T unflooded	-1.7235	3.84	188	-0.449	0.9998
TA flooded	-	T flooded	3.2224	3.04	188	1.059	0.9642
TA flooded	-	PT unflooded	-3.5223	3.55	188	-0.993	0.975
TA flooded	-	PTA unflooded	-8.2649	4.33	188	-1.91	0.546
TA flooded	-	TA unflooded	-1.1742	3.55	188	-0.331	1

TA flooded	-	T unflooded	2.7003	3.15	188	0.858	0.9893
T flooded	-	PT unflooded	-6.7447	2.99	188	-2.253	0.3255
T flooded	-	PTA unflooded	-11.4873	3.89	188	-2.955	0.0677
T flooded	-	TA unflooded	-4.3966	2.99	188	-1.469	0.8231
T flooded	-	T unflooded	-0.5221	2.51	188	-0.208	1
PT unflooded	-	PTA unflooded	-4.7426	4.29	188	-1.104	0.9552
PT unflooded	-	TA unflooded	2.3481	3.51	188	0.67	0.9977
PT unflooded	-	T unflooded	6.2226	3.1	188	2.007	0.48
PTA unflooded	-	TA unflooded	7.0907	4.29	188	1.651	0.7183
PTA unflooded	-	T unflooded	10.9652	3.97	188	2.762	0.1111
TA unflooded	-	T unflooded	3.8745	3.1	188	1.25	0.9157

Pot level top down surface area

Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	-128.41	30.2	87	-4.258	0.0039
A flooded	-	P flooded	-27.83	29.1	87	-0.955	0.9995
A flooded	-	PT Flooded	-59.63	29.1	87	-2.046	0.7342
A flooded	-	PTA flooded	-79.38	30.2	87	-2.632	0.3363
A flooded	-	TA flooded	-93.64	29.1	87	-3.214	0.0943
A flooded	-	T flooded	-44.9	30.2	87	-1.489	0.9662
A flooded	-	A unflooded	-63.09	30.2	87	-2.092	0.7045
A flooded	-	PA unflooded	-42.04	29.1	87	-1.443	0.9737
A flooded	-	P unflooded	-61.13	31.5	87	-1.942	0.7973
A flooded	-	PT unflooded	-47.56	30.2	87	-1.577	0.9474
A flooded	-	PTA unflooded	-77.38	30.2	87	-2.566	0.3774
A flooded	-	TA unflooded	-105.43	30.2	87	-3.496	0.044
A flooded	-	T unflooded	-80.42	31.5	87	-2.555	0.384
PA flooded	-	P flooded	100.58	30.2	87	3.335	0.0687
PA flooded	-	PT Flooded	68.78	30.2	87	2.281	0.5733
PA flooded	-	PTA flooded	49.03	31.1	87	1.574	0.9482
PA flooded	-	TA flooded	34.76	30.2	87	1.153	0.9965
PA flooded	-	T flooded	83.51	31.1	87	2.681	0.3076
PA flooded	-	A unflooded	65.32	31.1	87	2.097	0.7012
PA flooded	-	PA unflooded	86.37	30.2	87	2.864	0.214
PA flooded	-	P unflooded	67.28	32.4	87	2.075	0.7156
PA flooded	-	PT unflooded	80.85	31.1	87	2.596	0.3586
PA flooded	-	PTA unflooded	51.03	31.1	87	1.638	0.9307
PA flooded	-	TA unflooded	22.98	31.1	87	0.738	1
PA flooded	-	T unflooded	47.99	32.4	87	1.48	0.9677
P flooded	-	PT Flooded	-31.8	29.1	87	-1.091	0.998

P flooded	-	PTA flooded	-51.55	30.2	87	-1.709	0.907
P flooded	-	TA flooded	-65.81	29.1	87	-2.259	0.5889
P flooded	-	T flooded	-17.06	30.2	87	-0.566	1
P flooded	-	A unflooded	-35.26	30.2	87	-1.169	0.996
P flooded	-	PA unflooded	-14.21	29.1	87	-0.488	1
P flooded	-	P unflooded	-33.3	31.5	87	-1.058	0.9985
P flooded	-	PT unflooded	-19.73	30.2	87	-0.654	1
P flooded	-	PTA unflooded	-49.55	30.2	87	-1.643	0.9292
P flooded	-	TA unflooded	-77.6	30.2	87	-2.573	0.3729
P flooded	-	T unflooded	-52.59	31.5	87	-1.671	0.9203
PT Flooded	-	PTA flooded	-19.76	30.2	87	-0.655	1
PT Flooded	-	TA flooded	-34.02	29.1	87	-1.167	0.996
PT Flooded	-	T flooded	14.73	30.2	87	0.488	1
PT Flooded	-	A unflooded	-3.46	30.2	87	-0.115	1
PT Flooded	-	PA unflooded	17.58	29.1	87	0.603	1
PT Flooded	-	P unflooded	-1.51	31.5	87	-0.048	1
PT Flooded	-	PT unflooded	12.07	30.2	87	0.4	1
PT Flooded	-	PTA unflooded	-17.75	30.2	87	-0.589	1
PT Flooded	-	TA unflooded	-45.8	30.2	87	-1.519	0.9605
PT Flooded	-	T unflooded	-20.79	31.5	87	-0.661	1
PTA flooded	-	TA flooded	-14.26	30.2	87	-0.473	1
PTA flooded	-	T flooded	34.49	31.1	87	1.107	0.9976
PTA flooded	-	A unflooded	16.29	31.1	87	0.523	1
PTA flooded	-	PA unflooded	37.34	30.2	87	1.238	0.9931
PTA flooded	-	P unflooded	18.25	32.4	87	0.563	1
PTA flooded	-	PT unflooded	31.83	31.1	87	1.022	0.9989
PTA flooded	-	PTA unflooded	2	31.1	87	0.064	1
PTA flooded	-	TA unflooded	-26.04	31.1	87	-0.836	0.9999
PTA flooded	-	T unflooded	-1.04	32.4	87	-0.032	1
TA flooded	-	T flooded	48.75	30.2	87	1.616	0.937
TA flooded	-	A unflooded	30.55	30.2	87	1.013	0.999
TA flooded	-	PA unflooded	51.6	29.1	87	1.771	0.8828
TA flooded	-	P unflooded	32.51	31.5	87	1.033	0.9988
TA flooded	-	PT unflooded	46.09	30.2	87	1.528	0.9586
TA flooded	-	PTA unflooded	16.26	30.2	87	0.539	1
TA flooded	-	TA unflooded	-11.78	30.2	87	-0.391	1
TA flooded	-	T unflooded	13.22	31.5	87	0.42	1
T flooded	-	A unflooded	-18.2	31.1	87	-0.584	1
T flooded	-	PA unflooded	2.85	30.2	87	0.095	1
T flooded	-	P unflooded	-16.24	32.4	87	-0.501	1
T flooded	-	PT unflooded	-2.66	31.1	87	-0.085	1
T flooded	-	PTA unflooded	-32.49	31.1	87	-1.043	0.9987
T flooded	-	TA unflooded	-60.53	31.1	87	-1.943	0.7968
T flooded	-	T unflooded	-35.53	32.4	87	-1.096	0.9979
A unflooded	-	PA unflooded	21.05	30.2	87	0.698	1
A unflooded	-	P unflooded	1.96	32.4	87	0.06	1
A unflooded	-	PT unflooded	15.53	31.1	87	0.499	1
A unflooded	-	PTA unflooded	-14.29	31.1	87	-0.459	1
A unflooded	-	TA unflooded	-42.34	31.1	87	-1.359	0.9841

A unflooded	-	T unflooded	-17.33	32.4	87	-0.535	1
PA unflooded	-	P unflooded	-19.09	31.5	87	-0.607	1
PA unflooded	-	PT unflooded	-5.51	30.2	87	-0.183	1
PA unflooded	-	PTA unflooded	-35.34	30.2	87	-1.172	0.9959
PA unflooded	-	TA unflooded	-63.38	30.2	87	-2.102	0.6981
PA unflooded	-	T unflooded	-38.38	31.5	87	-1.219	0.994
P unflooded	-	PT unflooded	13.58	32.4	87	0.419	1
P unflooded	-	PTA unflooded	-16.25	32.4	87	-0.501	1
P unflooded	-	TA unflooded	-44.29	32.4	87	-1.366	0.9834
P unflooded	-	T unflooded	-19.29	33.6	87	-0.573	1
PT unflooded	-	PTA unflooded	-29.82	31.1	87	-0.957	0.9995
PT unflooded	-	TA unflooded	-57.87	31.1	87	-1.858	0.8427
PT unflooded	-	T unflooded	-32.86	32.4	87	-1.014	0.999
PTA unflooded	-	TA unflooded	-28.05	31.1	87	-0.9	0.9997
PTA unflooded	-	T unflooded	-3.04	32.4	87	-0.094	1
TA unflooded	-	T unflooded	25.01	32.4	87	0.771	1

Pot level side on surface area							
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	-113.99	55.5	91	-2.053	0.7302
A flooded	-	P flooded	-95.27	53.6	91	-1.776	0.8809
A flooded	-	PT Flooded	-41.55	53.6	91	-0.775	0.9999
A flooded	-	PTA flooded	-227.78	53.6	91	-4.246	0.0039
A flooded	-	TA flooded	-192.28	57.9	91	-3.319	0.071
A flooded	-	T flooded	-5.3	53.6	91	-0.099	1
A flooded	-	A unflooded	-119.08	53.6	91	-2.22	0.6163
A flooded	-	PA unflooded	-76.69	57.9	91	-1.324	0.9874
A flooded	-	P unflooded	24.22	55.5	91	0.436	1
A flooded	-	PT unflooded	-9.11	53.6	91	-0.17	1
A flooded	-	PTA unflooded	-131	55.5	91	-2.359	0.5171
A flooded	-	TA unflooded	-214.61	53.6	91	-4.001	0.009
A flooded	-	T unflooded	-88.2	53.6	91	-1.644	0.929
PA flooded	-	P flooded	18.72	55.5	91	0.337	1
PA flooded	-	PT Flooded	72.44	55.5	91	1.305	0.9889
PA flooded	-	PTA flooded	-113.79	55.5	91	-2.049	0.7325
PA flooded	-	TA flooded	-78.3	59.7	91	-1.312	0.9884
PA flooded	-	T flooded	108.69	55.5	91	1.958	0.7888
PA flooded	-	A unflooded	-5.09	55.5	91	-0.092	1
PA flooded	-	PA unflooded	37.29	59.7	91	0.625	1
PA flooded	-	P unflooded	138.21	57.3	91	2.41	0.4813
PA flooded	-	PT unflooded	104.88	55.5	91	1.889	0.827
PA flooded	-	PTA unflooded	-17.01	57.3	91	-0.297	1

PA flooded	-	TA unflooded	-100.62	55.5	91	-1.812	0.8648
PA flooded	-	T unflooded	25.78	55.5	91	0.464	1
P flooded	-	PT Flooded	53.72	53.6	91	1.001	0.9992
P flooded	-	PTA flooded	-132.51	53.6	91	-2.47	0.4398
P flooded	-	TA flooded	-97.02	57.9	91	-1.675	0.9194
P flooded	-	T flooded	89.97	53.6	91	1.677	0.9185
P flooded	-	A unflooded	-23.81	53.6	91	-0.444	1
P flooded	-	PA unflooded	18.57	57.9	91	0.321	1
P flooded	-	P unflooded	119.49	55.5	91	2.152	0.6638
P flooded	-	PT unflooded	86.16	53.6	91	1.606	0.94
P flooded	-	PTA unflooded	-35.73	55.5	91	-0.644	1
P flooded	-	TA unflooded	-119.34	53.6	91	-2.225	0.6128
P flooded	-	T unflooded	7.06	53.6	91	0.132	1
PT Flooded	-	PTA flooded	-186.23	53.6	91	-3.472	0.0465
PT Flooded	-	TA flooded	-150.74	57.9	91	-2.602	0.3542
PT Flooded	-	T flooded	36.25	53.6	91	0.676	1
PT Flooded	-	A unflooded	-77.53	53.6	91	-1.445	0.9735
PT Flooded	-	PA unflooded	-35.15	57.9	91	-0.607	1
PT Flooded	-	P unflooded	65.77	55.5	91	1.185	0.9955
PT Flooded	-	PT unflooded	32.44	53.6	91	0.605	1
PT Flooded	-	PTA unflooded	-89.45	55.5	91	-1.611	0.9387
PT Flooded	-	TA unflooded	-173.06	53.6	91	-3.226	0.0906
PT Flooded	-	T unflooded	-46.66	53.6	91	-0.87	0.9998
PTA flooded	-	TA flooded	35.49	57.9	91	0.613	1
PTA flooded	-	T flooded	222.48	53.6	91	4.148	0.0055
PTA flooded	-	A unflooded	108.7	53.6	91	2.026	0.7471
PTA flooded	-	PA unflooded	151.08	57.9	91	2.608	0.3505
PTA flooded	-	P unflooded	252	55.5	91	4.539	0.0014
PTA flooded	-	PT unflooded	218.67	53.6	91	4.077	0.007
PTA flooded	-	PTA unflooded	96.78	55.5	91	1.743	0.8945
PTA flooded	-	TA unflooded	13.17	53.6	91	0.245	1
PTA flooded	-	T unflooded	139.57	53.6	91	2.602	0.354
TA flooded	-	T flooded	186.99	57.9	91	3.227	0.0904
TA flooded	-	A unflooded	73.21	57.9	91	1.264	0.9917
TA flooded	-	PA unflooded	115.59	61.9	91	1.866	0.8387
TA flooded	-	P unflooded	216.51	59.7	91	3.628	0.0295
TA flooded	-	PT unflooded	183.18	57.9	91	3.162	0.1068
TA flooded	-	PTA unflooded	61.29	59.7	91	1.027	0.9989
TA flooded	-	TA unflooded	-22.32	57.9	91	-0.385	1
TA flooded	-	T unflooded	104.08	57.9	91	1.796	0.872
T flooded	-	A unflooded	-113.78	53.6	91	-2.121	0.685
T flooded	-	PA unflooded	-71.4	57.9	91	-1.232	0.9934
T flooded	-	P unflooded	29.52	55.5	91	0.532	1
T flooded	-	PT unflooded	-3.81	53.6	91	-0.071	1
T flooded	-	PTA unflooded	-125.7	55.5	91	-2.264	0.585
T flooded	-	TA unflooded	-209.31	53.6	91	-3.902	0.0125
T flooded	-	T unflooded	-82.91	53.6	91	-1.546	0.955
A unflooded	-	PA unflooded	42.38	57.9	91	0.731	1
A unflooded	-	P unflooded	143.3	55.5	91	2.581	0.3672

A unflooded	-	PT unflooded	109.97	53.6	91	2.05	0.732
A unflooded	-	PTA unflooded	-11.92	55.5	91	-0.215	1
A unflooded	-	TA unflooded	-95.53	53.6	91	-1.781	0.8788
A unflooded	-	T unflooded	30.87	53.6	91	0.576	1
PA unflooded	-	P unflooded	100.92	59.7	91	1.691	0.9139
PA unflooded	-	PT unflooded	67.59	57.9	91	1.167	0.9961
PA unflooded	-	PTA unflooded	-54.3	59.7	91	-0.91	0.9997
PA unflooded	-	TA unflooded	-137.91	57.9	91	-2.38	0.5022
PA unflooded	-	T unflooded	-11.51	57.9	91	-0.199	1
P unflooded	-	PT unflooded	-33.33	55.5	91	-0.6	1
P unflooded	-	PTA unflooded	-155.22	57.3	91	-2.707	0.2923
P unflooded	-	TA unflooded	-238.83	55.5	91	-4.302	0.0032
P unflooded	-	T unflooded	-112.43	55.5	91	-2.025	0.748
PT unflooded	-	PTA unflooded	-121.89	55.5	91	-2.195	0.6337
PT unflooded	-	TA unflooded	-205.5	53.6	91	-3.831	0.0157
PT unflooded	-	T unflooded	-79.09	53.6	91	-1.475	0.9688
PTA unflooded	-	TA unflooded	-83.61	55.5	91	-1.506	0.9632
PTA unflooded	-	T unflooded	42.79	55.5	91	0.771	1
TA unflooded	-	T unflooded	126.4	53.6	91	2.357	0.5191

	Pot level above ground biomass						
Contrast			Estimate	SE		df	T ratio	P Value
A flooded	-	PA flooded	4.649	7.33		72	0.634	1
A flooded	-	P flooded	-10.086	6.95		72	-1.452	0.9716
A flooded	-	PT Flooded	-25.23	6.43		72	-3.924	0.0132
A flooded	-	PTA flooded	-7.869	6.95		72	-1.133	0.9969
A flooded	-	TA flooded	4.395	7.33		72	0.599	1
A flooded	-	T flooded	-9.458	6.66		72	-1.421	0.9762
A flooded	-	A unflooded	-3.399	6.95		72	-0.489	1
A flooded	-	PA unflooded	-5.58	6.95		72	-0.803	0.9999
A flooded	-	P unflooded	-13.579	6.43		72	-2.112	0.6909
A flooded	-	PT unflooded	-19.999	6.66		72	-3.005	0.1608
A flooded	-	PTA unflooded	1.975	7.33		72	0.269	1
A flooded	-	TA unflooded	3.427	7.33		72	0.467	1

A flooded	-	T unflooded	-5.179	7.88		72	-0.658	1
PA flooded	-	P flooded	-14.735	7.79		72	-1.892	0.8237
PA flooded	-	PT Flooded	-29.879	7.33		72	-4.076	0.0081
PA flooded	-	PTA flooded	-12.518	7.79		72	-1.607	0.9384
PA flooded	-	TA flooded	-0.254	8.13		72	-0.031	1
PA flooded	-	T flooded	-14.106	7.53		72	-1.873	0.8336
PA flooded	-	A unflooded	-8.048	7.79		72	-1.033	0.9988
PA flooded	-	PA unflooded	-10.229	7.79		72	-1.314	0.9878
PA flooded	-	P unflooded	-18.228	7.33		72	-2.486	0.4318
PA flooded	-	PT unflooded	-24.647	7.53		72	-3.273	0.0844
PA flooded	-	PTA unflooded	-2.674	8.13		72	-0.329	1
PA flooded	-	TA unflooded	-1.222	8.13		72	-0.15	1
PA flooded	-	T unflooded	-9.828	8.63		72	-1.139	0.9967
P flooded	-	PT Flooded	-15.144	6.95		72	-2.181	0.6441
P flooded	-	PTA flooded	2.218	7.42		72	0.299	1
P flooded	-	TA flooded	14.481	7.79		72	1.86	0.8405
P flooded	-	T flooded	0.629	7.15		72	0.088	1
P flooded	-	A unflooded	6.687	7.42		72	0.901	0.9997
P flooded	-	PA unflooded	4.506	7.42		72	0.607	1
P flooded	-	P unflooded	-3.492	6.95		72	-0.503	1
P flooded	-	PT unflooded	-9.912	7.15		72	-1.385	0.9807
P flooded	-	PTA unflooded	12.061	7.79		72	1.549	0.9531
P flooded	-	TA unflooded	13.513	7.79		72	1.735	0.896
P flooded	-	T unflooded	4.907	8.3		72	0.591	1
PT Flooded	-	PTA flooded	17.362	6.95		72	2.5	0.4228
PT Flooded	-	TA flooded	29.625	7.33		72	4.041	0.0091
PT Flooded	-	T flooded	15.773	6.66		72	2.37	0.5114
PT Flooded	-	A unflooded	21.831	6.95		72	3.143	0.1165
PT Flooded	-	PA unflooded	19.65	6.95		72	2.829	0.2339
PT Flooded	-	P unflooded	11.652	6.43		72	1.812	0.8634

PT Flooded	-	PT unflooded	5.232	6.66		72	0.786	0.9999
PT Flooded	-	PTA unflooded	27.206	7.33		72	3.711	0.0252
PT Flooded	-	TA unflooded	28.657	7.33		72	3.909	0.0138
PT Flooded	-	T unflooded	20.051	7.88		72	2.546	0.3927
PTA flooded	-	TA flooded	12.264	7.79		72	1.575	0.947
PTA flooded	-	T flooded	-1.589	7.15		72	-0.222	1
PTA flooded	-	A unflooded	4.47	7.42		72	0.602	1
PTA flooded	-	PA unflooded	2.288	7.42		72	0.308	1
PTA flooded	-	P unflooded	-5.71	6.95		72	-0.822	0.9999
PTA flooded	-	PT unflooded	-12.13	7.15		72	-1.695	0.9108
PTA flooded	-	PTA unflooded	9.844	7.79		72	1.264	0.9913
PTA flooded	-	TA unflooded	11.295	7.79		72	1.451	0.9719
PTA flooded	-	T unflooded	2.69	8.3		72	0.324	1
TA flooded	-	T flooded	-13.853	7.53		72	-1.84	0.8503
TA flooded	-	A unflooded	-7.794	7.79		72	-1.001	0.9991
TA flooded	-	PA unflooded	-9.975	7.79		72	-1.281	0.9902
TA flooded	-	P unflooded	-17.974	7.33		72	-2.452	0.455
TA flooded	-	PT unflooded	-24.394	7.53		72	-3.24	0.0919
TA flooded	-	PTA unflooded	-2.42	8.13		72	-0.298	1
TA flooded	-	TA unflooded	-0.969	8.13		72	-0.119	1
TA flooded	-	T unflooded	-9.574	8.63		72	-1.11	0.9975
T flooded	-	A unflooded	6.059	7.15		72	0.847	0.9999
T flooded	-	PA unflooded	3.877	7.15		72	0.542	1
T flooded	-	P unflooded	-4.121	6.66		72	-0.619	1
T flooded	-	PT unflooded	-10.541	6.87		72	-1.533	0.9566
T flooded	-	PTA unflooded	11.433	7.53		72	1.518	0.9597
T flooded	-	TA unflooded	12.884	7.53		72	1.711	0.9052

T flooded	-	T unflooded	4.278	8.06		72	0.531	1
A unflooded	-	PA unflooded	-2.181	7.42		72	-0.294	1
A unflooded	-	P unflooded	-10.18	6.95		72	-1.466	0.9694
A unflooded	-	PT unflooded	-16.6	7.15		72	-2.32	0.5462
A unflooded	-	PTA unflooded	5.374	7.79		72	0.69	1
A unflooded	-	TA unflooded	6.826	7.79		72	0.877	0.9998
A unflooded	-	T unflooded	-1.78	8.3		72	-0.214	1
PA unflooded	-	P unflooded	-7.998	6.95		72	-1.152	0.9964
PA unflooded	-	PT unflooded	-14.418	7.15		72	-2.015	0.753
PA unflooded	-	PTA unflooded	7.556	7.79		72	0.97	0.9994
PA unflooded	-	TA unflooded	9.007	7.79		72	1.157	0.9962
PA unflooded	-	T unflooded	0.401	8.3		72	0.048	1
P unflooded	-	PT unflooded	-6.42	6.66		72	-0.965	0.9994
P unflooded	-	PTA unflooded	15.554	7.33		72	2.122	0.6843
P unflooded	-	TA unflooded	17.005	7.33		72	2.32	0.5466
P unflooded	-	T unflooded	8.4	7.88		72	1.067	0.9983
PT unflooded	-	PTA unflooded	21.974	7.53		72	2.918	0.1944
PT unflooded	-	TA unflooded	23.425	7.53		72	3.111	0.1259
PT unflooded	-	T unflooded	14.82	8.06		72	1.839	0.8509
PTA unflooded	-	TA unflooded	1.451	8.13		72	0.178	1
PTA unflooded	-	T unflooded	-7.154	8.63		72	-0.829	0.9999
TA unflooded	-	T unflooded	-8.606	8.63		72	-0.998	0.9991

Pot level root mass						
Contrast			Estimate	SE	df	T ratio	P Value
A flooded	-	PA flooded	-2.82336	4.96	82	-0.569	1

A flooded	-	P flooded	-14.7873	5.18	82	-2.854	0.2194
A flooded	-	PT Flooded	-12.4497	4.96	82	-2.508	0.4159
A flooded	-	PTA flooded	-3.79692	4.96	82	-0.765	1
A flooded	-	TA flooded	-2.81692	5.18	82	-0.544	1
A flooded	-	T flooded	-0.99852	5.18	82	-0.193	1
A flooded	-	A unflooded	-9.90931	4.8	82	-2.066	0.7214
A flooded	-	PA unflooded	-8.28073	4.8	82	-1.726	0.9003
A flooded	-	P unflooded	-7.74764	4.8	82	-1.615	0.937
A flooded	-	PT unflooded	-23.62	4.96	82	-4.758	0.0007
A flooded	-	PTA unflooded	-10.3806	4.96	82	-2.091	0.705
A flooded	-	TA unflooded	-0.16787	5.18	82	-0.032	1
A flooded	-	T unflooded	-9.61693	5.47	82	-1.759	0.8876
PA flooded	-	P flooded	-11.9639	5.34	82	-2.242	0.6011
PA flooded	-	PT Flooded	-9.62636	5.13	82	-1.877	0.8323
PA flooded	-	PTA flooded	-0.97356	5.13	82	-0.19	1
PA flooded	-	TA flooded	0.00645	5.34	82	0.001	1
PA flooded	-	T flooded	1.82484	5.34	82	0.342	1
PA flooded	-	A unflooded	-7.08595	4.96	82	-1.427	0.9758
PA flooded	-	PA unflooded	-5.45736	4.96	82	-1.099	0.9978
PA flooded	-	P unflooded	-4.92427	4.96	82	-0.992	0.9992
PA flooded	-	PT unflooded	-20.7967	5.13	82	-4.056	0.008
PA flooded	-	PTA unflooded	-7.55728	5.13	82	-1.474	0.9686
PA flooded	-	TA unflooded	2.6555	5.34	82	0.498	1
PA flooded	-	T unflooded	-6.79357	5.62	82	-1.209	0.9944
P flooded	-	PT Flooded	2.33753	5.34	82	0.438	1
P flooded	-	PTA flooded	10.99033	5.34	82	2.059	0.7257
P flooded	-	TA flooded	11.97033	5.54	82	2.161	0.6573
P flooded	-	T flooded	13.78873	5.54	82	2.49	0.4279
P flooded	-	A unflooded	4.87794	5.18	82	0.942	0.9995
P flooded	-	PA unflooded	6.50652	5.18	82	1.256	0.992
P flooded	-	P unflooded	7.03961	5.18	82	1.359	0.984
P flooded	-	PT unflooded	-8.83279	5.34	82	-1.655	0.9251
P flooded	-	PTA unflooded	4.40661	5.34	82	0.826	0.9999
P flooded	-	TA unflooded	14.61938	5.54	82	2.64	0.3327
P flooded	-	T unflooded	5.17032	5.81	82	0.89	0.9998
PT Flooded	-	PTA flooded	8.6528	5.13	82	1.688	0.9144
PT Flooded	-	TA flooded	9.6328	5.34	82	1.805	0.8675
PT Flooded	-	T flooded	11.4512	5.34	82	2.146	0.6681
PT Flooded	-	A unflooded	2.54041	4.96	82	0.512	1
PT Flooded	-	PA unflooded	4.169	4.96	82	0.84	0.9999
PT Flooded	-	P unflooded	4.70208	4.96	82	0.947	0.9995
PT Flooded	-	PT unflooded	-11.1703	5.13	82	-2.179	0.6455
PT Flooded	-	PTA unflooded	2.06908	5.13	82	0.404	1
PT Flooded	-	TA unflooded	12.28186	5.34	82	2.301	0.5588
PT Flooded	-	T unflooded	2.83279	5.62	82	0.504	1
PTA flooded	-	TA flooded	0.98001	5.34	82	0.184	1
PTA flooded	-	T flooded	2.7984	5.34	82	0.524	1
PTA flooded	-	A unflooded	-6.11239	4.96	82	-1.231	0.9934
PTA flooded	-	PA unflooded	-4.4838	4.96	82	-0.903	0.9997

PTA flooded	-	P unflooded	-3.95072	4.96	82	-0.796	0.9999
PTA flooded	-	PT unflooded	-19.8231	5.13	82	-3.866	0.0147
PTA flooded	-	PTA unflooded	-6.58372	5.13	82	-1.284	0.9903
PTA flooded	-	TA unflooded	3.62906	5.34	82	0.68	1
PTA flooded	-	T unflooded	-5.82001	5.62	82	-1.036	0.9988
TA flooded	-	T flooded	1.81839	5.54	82	0.328	1
TA flooded	-	A unflooded	-7.0924	5.18	82	-1.369	0.9829
TA flooded	-	PA unflooded	-5.46381	5.18	82	-1.055	0.9985
TA flooded	-	P unflooded	-4.93072	5.18	82	-0.952	0.9995
TA flooded	-	PT unflooded	-20.8031	5.34	82	-3.898	0.0133
TA flooded	-	PTA unflooded	-7.56372	5.34	82	-1.417	0.9771
TA flooded	-	TA unflooded	2.64905	5.54	82	0.478	1
TA flooded	-	T unflooded	-6.80001	5.81	82	-1.171	0.9959
T flooded	-	A unflooded	-8.91079	5.18	82	-1.72	0.9027
T flooded	-	PA unflooded	-7.2822	5.18	82	-1.406	0.9787
T flooded	-	P unflooded	-6.74911	5.18	82	-1.303	0.9889
T flooded	-	PT unflooded	-22.6215	5.34	82	-4.239	0.0043
T flooded	-	PTA unflooded	-9.38212	5.34	82	-1.758	0.8878
T flooded	-	TA unflooded	0.83066	5.54	82	0.15	1
T flooded	-	T unflooded	-8.61841	5.81	82	-1.484	0.9669
A unflooded	-	PA unflooded	1.62859	4.8	82	0.34	1
A unflooded	-	P unflooded	2.16167	4.8	82	0.451	1
A unflooded	-	PT unflooded	-13.7107	4.96	82	-2.762	0.2647
A unflooded	-	PTA unflooded	-0.47133	4.96	82	-0.095	1
A unflooded	-	TA unflooded	9.74145	5.18	82	1.88	0.8308
A unflooded	-	T unflooded	0.29238	5.47	82	0.053	1
PA unflooded	-	P unflooded	0.53309	4.8	82	0.111	1
PA unflooded	-	PT unflooded	-15.3393	4.96	82	-3.09	0.1295
PA unflooded	-	PTA unflooded	-2.09992	4.96	82	-0.423	1
PA unflooded	-	TA unflooded	8.11286	5.18	82	1.566	0.9498
PA unflooded	-	T unflooded	-1.33621	5.47	82	-0.244	1
P unflooded	-	PT unflooded	-15.8724	4.96	82	-3.197	0.0995
P unflooded	-	PTA unflooded	-2.633	4.96	82	-0.53	1
P unflooded	-	TA unflooded	7.57977	5.18	82	1.463	0.9704
P unflooded	-	T unflooded	-1.86929	5.47	82	-0.342	1
PT unflooded	-	PTA unflooded	13.23939	5.13	82	2.582	0.3679
PT unflooded	-	TA unflooded	23.45217	5.34	82	4.394	0.0025
PT unflooded	-	T unflooded	14.00311	5.62	82	2.493	0.4256
PTA unflooded	-	TA unflooded	10.21278	5.34	82	1.914	0.8131
PTA unflooded	-	T unflooded	0.76371	5.62	82	0.136	1
TA unflooded	-	T unflooded	-9.44906	5.81	82	-1.627	0.9337

Pot level total biomass					
Contrast	Estimate	SE	df	T ratio	P Value

A flooded	-	PA flooded	1.062	8.62	64	0.123	1
A flooded	-	P flooded	-30.307	9.26	64	-3.273	0.0871
A flooded	-	PT Flooded	-37.918	7.83	64	-4.845	0.0007
A flooded	-	PTA flooded	-8.326	8.62	64	-0.966	0.9994
A flooded	-	TA flooded	3.266	9.26	64	0.353	1
A flooded	-	T flooded	-9.389	8.17	64	-1.15	0.9963
A flooded	-	A unflooded	-9.74	8.17	64	-1.193	0.9948
A flooded	-	PA unflooded	-10.09	8.17	64	-1.235	0.9928
A flooded	-	P unflooded	-21.326	7.56	64	-2.82	0.2409
A flooded	-	PT unflooded	-43.619	7.83	64	-5.573	<.0001
A flooded	-	PTA unflooded	-8.62	8.62	64	-1	0.9991
A flooded	-	TA unflooded	6.763	10.24	64	0.661	1
A flooded	-	T unflooded	-10.83	9.26	64	-1.169	0.9957
PA flooded	-	P flooded	-31.369	10.14	64	-3.092	0.1344
PA flooded	-	PT Flooded	-38.98	8.85	64	-4.402	0.003
PA flooded	-	PTA flooded	-9.388	9.56	64	-0.982	0.9992
PA flooded	-	TA flooded	2.204	10.14	64	0.217	1
PA flooded	-	T flooded	-10.451	9.16	64	-1.141	0.9966
PA flooded	-	A unflooded	-10.802	9.16	64	-1.18	0.9953
PA flooded	-	PA unflooded	-11.152	9.16	64	-1.218	0.9937
PA flooded	-	P unflooded	-22.388	8.62	64	-2.597	0.3629
PA flooded	-	PT unflooded	-44.681	8.85	64	-5.046	0.0003
PA flooded	-	PTA unflooded	-9.682	9.56	64	-1.012	0.999
PA flooded	-	TA unflooded	5.701	11.04	64	0.516	1
PA flooded	-	T unflooded	-11.892	10.14	64	-1.172	0.9956
P flooded	-	PT Flooded	-7.611	9.48	64	-0.803	0.9999
P flooded	-	PTA flooded	21.981	10.14	64	2.167	0.6535
P flooded	-	TA flooded	33.573	10.69	64	3.14	0.1203
P flooded	-	T flooded	20.918	9.76	64	2.143	0.6697
P flooded	-	A unflooded	20.567	9.76	64	2.107	0.6938
P flooded	-	PA unflooded	20.218	9.76	64	2.071	0.7172
P flooded	-	P unflooded	8.981	9.26	64	0.97	0.9993
P flooded	-	PT unflooded	-13.311	9.48	64	-1.404	0.978
P flooded	-	PTA unflooded	21.687	10.14	64	2.138	0.6731
P flooded	-	TA unflooded	37.07	11.55	64	3.21	0.1017
P flooded	-	T unflooded	19.477	10.69	64	1.821	0.8581
PT Flooded	-	PTA flooded	29.592	8.85	64	3.342	0.073
PT Flooded	-	TA flooded	41.184	9.48	64	4.345	0.0037
PT Flooded	-	T flooded	28.529	8.41	64	3.391	0.0643
PT Flooded	-	A unflooded	28.178	8.41	64	3.349	0.0717
PT Flooded	-	PA unflooded	27.829	8.41	64	3.308	0.0797
PT Flooded	-	P unflooded	16.592	7.83	64	2.12	0.6851
PT Flooded	-	PT unflooded	-5.7	8.08	64	-0.705	1
PT Flooded	-	PTA unflooded	29.299	8.85	64	3.309	0.0795
PT Flooded	-	TA unflooded	44.681	10.44	64	4.282	0.0045
PT Flooded	-	T unflooded	27.088	9.48	64	2.858	0.2235
PTA flooded	-	TA flooded	11.592	10.14	64	1.143	0.9965
PTA flooded	-	T flooded	-1.063	9.16	64	-0.116	1
PTA flooded	-	A unflooded	-1.414	9.16	64	-0.154	1

PTA flooded	-	PA unflooded	-1.764	9.16	64	-0.193	1
PTA flooded	-	P unflooded	-13	8.62	64	-1.508	0.9612
PTA flooded	-	PT unflooded	-35.293	8.85	64	-3.986	0.0117
PTA flooded	-	PTA unflooded	-0.294	9.56	64	-0.031	1
PTA flooded	-	TA unflooded	15.089	11.04	64	1.366	0.9825
PTA flooded	-	T unflooded	-2.504	10.14	64	-0.247	1
TA flooded	-	T flooded	-12.655	9.76	64	-1.296	0.9889
TA flooded	-	A unflooded	-13.006	9.76	64	-1.332	0.9859
TA flooded	-	PA unflooded	-13.356	9.76	64	-1.368	0.9823
TA flooded	-	P unflooded	-24.592	9.26	64	-2.656	0.3281
TA flooded	-	PT unflooded	-46.885	9.48	64	-4.946	0.0005
TA flooded	-	PTA unflooded	-11.886	10.14	64	-1.172	0.9956
TA flooded	-	TA unflooded	3.497	11.55	64	0.303	1
TA flooded	-	T unflooded	-14.096	10.69	64	-1.318	0.9872
T flooded	-	A unflooded	-0.351	8.73	64	-0.04	1
T flooded	-	PA unflooded	-0.701	8.73	64	-0.08	1
T flooded	-	P unflooded	-11.937	8.17	64	-1.462	0.9696
T flooded	-	PT unflooded	-34.23	8.41	64	-4.068	0.009
T flooded	-	PTA unflooded	0.769	9.16	64	0.084	1
T flooded	-	TA unflooded	16.152	10.69	64	1.51	0.9607
T flooded	-	T unflooded	-1.441	9.76	64	-0.148	1
A unflooded	-	PA unflooded	-0.349	8.73	64	-0.04	1
A unflooded	-	P unflooded	-11.586	8.17	64	-1.419	0.9761
A unflooded	-	PT unflooded	-33.878	8.41	64	-4.027	0.0103
A unflooded	-	PTA unflooded	1.12	9.16	64	0.122	1
A unflooded	-	TA unflooded	16.503	10.69	64	1.543	0.9538
A unflooded	-	T unflooded	-1.09	9.76	64	-0.112	1
PA unflooded	-	P unflooded	-11.237	8.17	64	-1.376	0.9815
PA unflooded	-	PT unflooded	-33.529	8.41	64	-3.985	0.0117
PA unflooded	-	PTA unflooded	1.47	9.16	64	0.161	1
PA unflooded	-	TA unflooded	16.852	10.69	64	1.576	0.946
PA unflooded	-	T unflooded	-0.74	9.76	64	-0.076	1
P unflooded	-	PT unflooded	-22.292	7.83	64	-2.848	0.2278
P unflooded	-	PTA unflooded	12.707	8.62	64	1.474	0.9675
P unflooded	-	TA unflooded	28.089	10.24	64	2.744	0.2794
P unflooded	-	T unflooded	10.496	9.26	64	1.133	0.9968
PT unflooded	-	PTA unflooded	34.999	8.85	64	3.953	0.0129
PT unflooded	-	TA unflooded	50.381	10.44	64	4.828	0.0007
PT unflooded	-	T unflooded	32.789	9.48	64	3.459	0.0537
PTA unflooded	-	TA unflooded	15.382	11.04	64	1.393	0.9795
PTA unflooded	-	T unflooded	-2.21	10.14	64	-0.218	1
TA unflooded	-	T unflooded	-17.593	11.55	64	-1.523	0.9581

Appendix 2.3

Table 2.3.1 Examples of variation within each species functional trait. UF mono is variation within the unflooded monocultures and all is the variation of the other treatments for each species combined

		Aster tripolium		*Plantago maritima*		*Triglochin maritima*	
		UF Mono	All	UF Mono	All	UF Mono	All
Height (mm)	25%	138	130	95	140	148	170
	Median	170	170	120	180	240	220
	75%	196	215	170	230	330	270
Width (mm)	25%	88	110	153	100	180	175
	Median	120	175	190	150	210	230
	75%	173	270	265	220	280	310
Number of leaves	25%	12	13	21	11	13	16
	Median	17	23	32	19	19	25
	75%	31	34	43	29	25	38
Total biomass (mg)	25%	3.90	2.67	4.92	2.32	3.36	3.98
	Median	5.34	5.36	8.04	4.57	5.80	6.57
	75%	7.68	8.47	10.80	7.53	8.46	10.99
Above ground biomass (mg)	25%	1.15	0.79	2.05	2.35	1.12	0.88
	Median	2.30	1.85	4.08	4.44	2.69	2.18
	75%	3.94	2.46	6.37	7.25	5.63	5.89
Root mass (mg)	25%	0.85	0.71	1.55	1.36	0.33	0.32
	Median	1.81	1.50	2.88	2.77	1.45	1.06
	75%	3.49	3.09	4.51	4.51	3.76	4.14
Total biomass (mg)	25%	3.26	2.24	4.92	4.93	3.30	2.55
	Median	4.66	4.18	7.28	7.90	5.38	5.27
	75%	7.04	6.50	10.72	11.72	9.18	9.07
Specific leaf area ($mm^2\ mg^{-1}$)	25%	10.99	6.27	5.33	7.98	5.67	6.58
	Median	14.82	10.02	7.68	11.70	7.67	8.71
	75%	23.99	15.42	10.11	17.97	11.71	12.89

Appendix 4.1

Table 4.1 Full lists of all chi squared and fishers exact test statistics for Survival response of different genetic identities of Festuca rubra and Puccinellia maritima to different elevation and nutrient conditions

Festuca rubra															
Elevation			**Nutrients**									**Genetic identitiy**			
x2 13.93	df 1	p 0.0002				x2 0.658	df 1	p 0.4172				x2 10.17	df 4	p 0.0377	
Genetic id 1			**Genetic id 2**			**Genetic id 3**			**Genetic id 4**			**Genetic id 5**			
Nutrients	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	
x2 2.008	x2 8.031		x2 0.127	x2 1.143		x2 1.309	x2 0.145		x2 0.502	x2 4.518					
df 1	df 1	p 0.002	df 1	df 1	p 0.002	df 1	df 1	p 0.168	df 1	df 1	p 0.084	p 0.172	p 2.273	p 0.084	
p 0.157	p 0.005		p 0.722	p 0.285		p 0.253	p 0.703		p 0.479	p 0.034					
Puccinellia Maritima															
Elevation			**Nutrients**									**Genetic identitiy**			
x2 0.153	df 1	p 0.696				x2 3.8177	df 1	p 0.051				x2 2.174	df 4	p 0.71	
Genetic id 1			**Genetic id 2**			**Genetic id 3**			**Genetic id 4**			**Genetic id 5**			
Nutrients	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	**Nutrients**	**Elevation**	**Elevation+Nutrients**	
p 0.519	p 1	p 0.942	p 0.44	p 1	p 0.942	p 0.6	p 1	p 0.886	p 1	p 1	p 0.234	p 0.002	p 0.685	p 0.007	